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Amines in Fish Muscle

I. Colorimetric Determination of Trimethylamine as the Picrate Salt

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(*Received for publication January 8, 1945*)

ABSTRACT

A sensitive accurate colorimetric method for trimethylamine determination is presented, based on the extraction with toluene of an alkaline sample containing 0.002 to 0.02 mg. trimethylamine nitrogen, and the formation of the yellow coloured picrate by mixing with a picric acid reagent. The application of the method in fishery products and effects of interfering substances have been investigated.

Trimethylamine estimation is the most satisfactory measure so far developed of spoilage in sea fish where the concentration of trimethylamine oxide is sufficient to provide an adequate source of trimethylamine. The time-consuming nature of present procedures and the special apparatus required limit their general use for routine determinations in the plant, or even in the research laboratory (Beatty and Gibbons 1937, Boury and Schvinte 1935). No colorimetric method has heretofore been proposed.

Trimethylamine is produced in spoiling fish muscle by the bacterial reduction of the trimethylamine oxide present, and its rise parallels that of the bacterial population. The bacterial growth takes place on the surface until well on in the spoilage cycle (Wood, Sigurdsson and Dyer 1942), and since composite samples of whole fish fillet are usually used, the method must be capable of measuring the very small initial increase in trimethylamine in order to give useful information of the onset of spoilage. Ammonia, and small amounts of monomethylamine and dimethylamine are also produced along with the trimethylamine. Choline, betaine, and γ butyrobetaine, containing trimethylamine radicals, also occur in fish muscle (Kutscher and Ackerman 1933), and these compounds must not interfere in the determination.

An examination of the literature showed a number of reactions for amines which might possibly be used for the estimation of trimethylamine.

REACTIONS FOR AMINES

The chloranil test for amines (Sivadjian 1931) gives different colours with primary, secondary and tertiary amines, but its sensitivity was found to be insufficient for the amine concentration found in fish extracts.

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When potassium ferrocyanide is added to a solution of choline and cobaltinitrite a deep green colour is produced (Jacobs and Hoffman 1931). It was found that trimethylamine could be substituted for the choline and that the resulting colour was related to the amount present. However the optimum conditions for quantitative results were not found.

The Folin aminoacid reagent, sodium naphthoquinone-4-sulphonate (Folin 1922, E. G. Schmidt 1938, Frame, Russel and Wilhelmi 1943), also was found to react with trimethylamine, but since ammonia and formaldehyde interfered, the test was unsatisfactory.

The phenol reagent of Folin and Ciocalteu (1927) is reduced by trimethylamine as well as phenols. No previous mention has been made of this reaction with trimethylamine, and since the test is used for protein decomposition in fish muscle (Bradley and Bailey 1940, Wood Sigurdsson and Dyer 1942), the effect of trimethylamine must be taken into account. The interference is negligible at low trimethylamine concentrations, but in spoiled fish containing 50 mg. trimethylamine nitrogen per 100 g. tissue, the colour due to trimethylamine equals that given by the products of protein decomposition. The reaction with trimethylamine is only moderately sensitive, since it produces but one twenty-sixth as much colour as tyrosine at equimolar concentration. Since distillation would be necessary to separate the protein decomposition products, the test was not considered an improvement over existing methods.

Finally, the amine reagent of Richter (1938) and Richter, Lee and Hill (1941) was adapted for use. These authors determined amines in urine and blood by extracting an alkaline solution with petroleum ether or toluene, separating the amines from lecithin, etc., by shaking in acid solution, and reextracting the amines with the solvent from the acid extract after making alkaline with potassium carbonate. A two per cent solution of picric acid in chloroform was then added to an aliquot of centrifuged extract diluted with an equal volume of chloroform, the resulting yellow colour being compared with standards.

The original method was found to be unnecessarily complicated and gave inaccurate results. Thorough investigation of the various factors, concentration, reagents, etc., led to the development of a highly satisfactory method.

THE PICRATE METHOD

Reagents

Toluene. C.p. reagent, dried by shaking with anhydrous sodium sulphate. Interfering substances may be removed if necessary by shaking with N sulphuric acid followed by distillation and drying with sodium sulphate.

Picric acid. 0.02 per cent solution of picric acid (dry), c.p., in moisture-free toluene. A 2 per cent stock solution may be conveniently prepared and the dilute reagent made as required. The latter should be practically colourless.

Potassium carbonate. 100 g. potassium carbonate, c.p., dissolved in 100 g. water.

Formaldehyde. 10 per cent solution of formalin (40 per cent formaldehyde, commercial, shaken with magnesium carbonate and filtered) in water.

Standard trimethylamine solution. Stock solution: 0.682 g. trimethylamine hydrochloride (Eastman Kodak Co.) and 1 ml. hydrochloric acid, c.p., dissolved in 100 ml. water. As required, a dilute standard is prepared by diluting 1 ml. stock solution and 1 ml. hydrochloric acid to 100 ml. This contains 0.01 mg. trimethylamine nitrogen per ml. and 1 or 2 ml. may be used conveniently for the standard colour solution.

Procedure

An aliquot of a sample solution containing 0.002 to 0.02 mg. nitrogen as trimethylamine is made to 4 ml. in a test tube. One ml. formaldehyde, 10 ml. toluene, and 3 ml. potassium carbonate solution are added. The tube is stoppered with a cellophane-covered cork and is shaken vigorously approximately 40 times. Five ml. of the toluene layer is pipetted off into a small test tube and 0.3 to 0.4 g. granular anhydrous sodium sulphate is added. This is shaken a few times to dry the toluene, which is then poured off into a dry colorimeter tube containing 5 ml. of the 0.02 per cent picric acid reagent. After mixing, the yellow colour is read in a photoelectric colorimeter using a filter with maximum transmission at 4200 ångstrom units, the blank being made similarly except that water is used instead of trimethylamine or test solution. It has been found convenient to prepare two blanks, using the first to clean pipettes, corks, etc., from chromogenic substances, and using the second in the colorimeter.

A standard curve may be made up by using the standard trimethylamine solution but it has been found more convenient to use a K value with the Evelyn colorimeter ($K = \frac{2 - \log G}{c}$, where G is the galvanometer reading when the blank is set at 100, that is per cent light transmission; c is the concentration expressed as mg. nitrogen per tube). In routine use, it is only necessary to check standards with new reagents.

Development of Method

solvents and alkali

Richter et al. (1941) used a 1 to 1 solution of chloroform and either petroleum ether or toluene in the final colour solution. We have found that the colour is almost as intense (90 per cent) if toluene is used throughout. The recovery of solvents by fractional distillation is thus avoided.

Toluene, petroleum ether, benzene and xylene all gave good results as extracting solvents, and toluene was adopted as being the most suitable. The solvent is recovered by shaking with a small volume of N sulphuric acid to remove amines, decanting, distilling, and drying with anhydrous sodium sulphate. Various ratios of alkali, solution and solvent were tried. The ratio of toluene to solution should be between 1 and 2 to avoid emulsion formation. Using 5 ml. test solution and 10 ml. toluene, 3 ml. potassium carbonate solution was necessary to give constant recovery. The alkaline carbonate liberates the amine from its salts, so that it passes into the toluene phase on shaking, the salts being insoluble in the solvent. The tubes should be shaken vigorously 30 to 40 times. A cello-

phane-covered cork should be used. The first blank cleans this cork and the pipettes, etc., from chromogenic substances. The toluene extract contains appreciable suspended water which may be conveniently removed by shaking a few times with 0.3 to 0.4 g. granular anhydrous sodium sulphate, added from the point of a spatula, followed by decantation. More complete and convenient removal of water was thus obtained than by centrifugation.

PICRIC ACID CONCENTRATION

The blanks obtained with the 0.08 per cent picric acid reagent as used by Richter et al. were quite coloured. Therefore various concentrations between 0.008 and 0.4 per cent were tried. From 0.01 to 0.05 per cent was found most

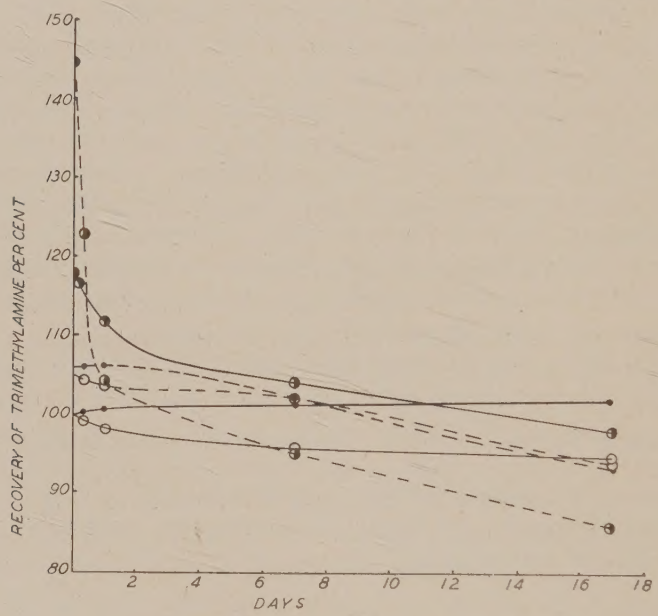

FIGURE 1 Effect of ammonia and formaldehyde on recovery of 0.01 mg. trimethylamine nitrogen. ———— Presence of formaldehyde, 1 ml 10 per cent solution. – – – No formaldehyde. ● 0.01 mg trimethylamine nitrogen; ○ 0.01 mg. trimethylamine nitrogen + 0.1 mg ammonia nitrogen; ◓ 0.01 mg trimethylamine nitrogen + 0.5 mg ammonia nitrogen

suitable, low recovery being obtained with concentrations greater than 0.1 per cent. The lower the concentration, the less is the error introduced by the blank due to the colour of the picric acid. A 0.02 per cent solution proved satisfactory. About 0.01 to 0.02 mg. of trimethylamine nitrogen per tube is usually present and the excess of picric acid is thus three to six times over the amount required to form the trimethylamine picrate salt, assuming maximum extraction of trimethylamine by the solvent.

AMMONIA AND FORMALDEHYDE

Ammonia gave cloudy solutions which could not be read in the colorimeter. The addition of 1 or 2 ml. of the 10 per cent formaldehyde reagent did not affect the recovery of trimethylamine, and was sufficient to eliminate the interference

of ammonia up to 0.2 mg. nitrogen, an excess of 10 to 20 times over the usual concentration of trimethylamine nitrogen. The formaldehyde itself gave a slight colour but the effect was eliminated when it was added to the blank tube. The effect of ammonia and of formaldehyde on the recovery of 0.01 mg. trimethylamine nitrogen up to 18 days is shown in figure 1.

Stability

The formaldehyde also increased the stability of the colour solution. If the tubes are stoppered to prevent evaporation, the colour is quite stable. Only a very small change in colour intensity occurred up to one month, particularly if the solution contained no ammonia. Thus colour standards for visual comparison may be kept for considerable periods, an important point in plant routine work.

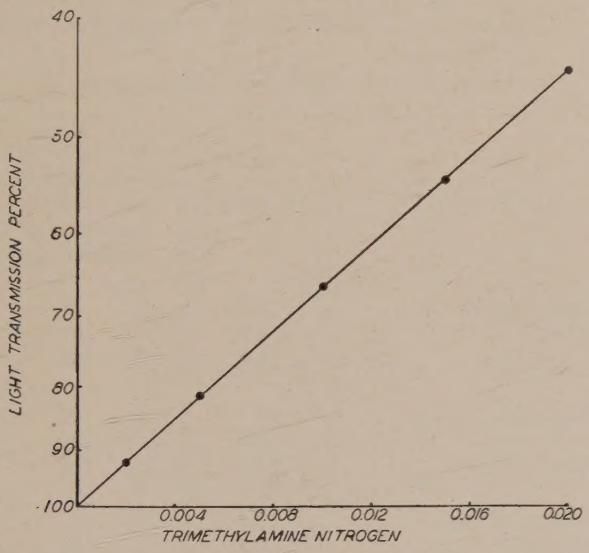

Figure 2. Standard curve for trimethylamine picrate. Per cent light transmission, logarithmic scale, plotted against mg. trimethylamine nitrogen per tube.

Standard Curves

A standard curve obtained by using various amounts of the standard trimethylamine solution is shown in figure 2. K values from a concentration of 0.002 to 0.020 mg. nitrogen per tube average 18.0 ($\pm$ 0.3), and Beer's law is followed over this range. Results from 35 points obtained on standard solutions over a period of three months under routine conditions gave similar results, with a slightly greater standard deviation, $K = 18.0$ ($\pm$ 0.9). Thus the reproducibility of the method is quite satisfactory.

Interferences

A solution containing 1 mg. nitrogen per tube of each of a number of the following amino acids and other substances were found to give no interference; glycine, asparagine, cysteine, cystine, tryptophane, histidine, tyrosine, gluta-

thione, creatine, creatinine, allantoin, urea, hydroxylamine, guanine, betaine, choline and trimethylamine oxide. Many of these are present in fish muscle and give picrate salts, but do not interfere because of insolubility in the toluene extractant. γ butyrobetaine, also present in fish (Kutscher and Ackerman 1933), was not tested but it probably behaves like betaine.

Lecithin gives a cloudy yellow colour. This probably explains the cloudy solutions obtained with extracts of fatty fish. No interference from this source occurred with cod or similar fish, although Kaucher et al. (1943) finds about three per cent lecithin in dried cod muscle. This interference may be eliminated when necessary, by a preliminary extraction with solvent from acid solution, under which condition trimethylamine is not extracted.

The primary, secondary and tertiary methylamines and ethylamines all react as shown in table I. With the exception of indole, the hydrochloride salts twice recrystallized from alcohol were tested. Only methylamine and dimethylamine

TABLE I. Colour values of various amines.

Compound	*K* value	Colour as per cent of colour given by an equivalent concentration of trimethylamine
Methylamine	2.00	11
Dimethylamine	3.78	21
Trimethylamine	18.0	100
Ethylamine	9.7	54
Diethylamine	18.9	105
Triethylamine	25.2	140
N-Propylamine	12.8	71
Histamine	5.4	30
Indole	0.07	0.4

are likely to be found in extracts from fish muscle so far as is known (Shewan 1939, Beatty and Collins 1940), and these only in small amounts, so that interference from this source is negligible. The trialkylamines give more colour than the di- or mono-derivatives, and the colour increases with length in the carbon chain, at least up to propylamine. Most of the higher aliphatic monoamines and various other amines have not been tested. These are not present in fresh tissue, but some are produced in advanced stages of spoilage by bacterial decarboxylation of aminoacids (C. L. A. Schmidt 1938, p. 236, Gale 1940, Richter et al. 1941, Gale and Epps 1944); for instance, histamine, indole, phenylethylamine, tyramine, cadaverine, and putrescine.

It was found that these nonvolatile amines were not produced in fish until spoilage was well advanced. Dyer and Mounsey (1945) demonstrated a difference between the colour values on the extract and on the distillate from the extract only when the trimethylamine values had reached 60 to 75 mg. nitrogen per 100 g. tissue. Fish are becoming unpalatable at levels of 10 to 15. Histamine gave 30

per cent of the colour of trimethylamine, and indole only 0.4 per cent. Richter et al. (1941) found that with his procedure, tyramine and putrescine did not give colours, while phenylethylamine yielded a colour about equal to trimethylamine. The presence of protein did not appreciably affect the recovery of trimethylamine in muscle extracts.

Comparison with Distillation Methods

The distillation methods of Beatty and Gibbons (1937), both the semimacrodistillation under reduced pressure and their modification of the Conway technique, were compared with the picrate procedure. Representative results obtained on fish extracts are given in table II.

Table II. Comparison of colorimetric and distillation methods.

Extract	Trimethylamine (mg. nitrogen per 100 g. muscle)				
	Colorimetric		Conway		Distillation (semi-macro)
Cod	0.20	$(\pm 0.04)_6$	0.22	$(\pm 0.05)_6$	
	0.84	$(\pm 0.1)_3$	0.80	$(\pm 0.1)_3$	
	2.7	$(\pm 0.2)_3$	2.7	$(\pm 0.1)_3$	
	11.2	$(\pm 0.3)_3$	10.8	$(\pm 1)_2$	
	33.5	$(\pm 0.5)_3$	34.3	$(\pm 0.5)_3$	
	90				73
	109	$(\pm 3)_3$	94	$(\pm 1)_3$	
Haddock	2.1	$(\pm 0.3)_3$	2.1	$(\pm 0.2)_3$	
	7.9	$(\pm 0.3)_3$	7.9	$(\pm 0.1)_3$	
	39.0	$(\pm 1)_2$	40.2	$(\pm 0.5)_2$	
	81				71
Smoked cod	11.0		9.8		11.1
	34.0		32.0		33.0
	34.3		33.5		
	93				93
Chicken haddie pickle	58.4		59.0		60.1
	28.0		30.0		27.0
Salt cod	76				78
Mackerel	4.5		2.4		2.5
	3.2		1.8		1.9

The results show very good agreement between all three methods. Since the colorimetric method is much shorter and is adaptable to the analysis of large numbers of routine tests, it is the most satisfactory for general use.

The method has been satisfactorily used on more than 8,000 estimations of trimethylamine on trichloracetic acid and formaldehyde extracts of fish and directly on the pickle from canned fish over a period of a year, and is now in use

in several laboratories. A simplified modification for use with visual comparator tubes, as given by Dyer (1943), is also in use in one plant laboratory. It should be noted, however, that a 0.02 per cent solution of picric acid in toluene is now used instead of a chloroform solution.

SUMMARY

A sensitive colorimetric method for estimating the spoilage of fish by trimethylamine determination has been developed. A sample containing 0.002 to 0.02 mg. nitrogen as trimethylamine is made alkaline with potassium carbonate in the presence of formaldehyde, extracted with toluene, and the dried extract mixed with a toluene solution of picric acid. The yellow colour of trimethylamine picrate is measured in the colorimeter. The colour is stable and obeys Beer's law over the range given.

Ammonia, aminoacids, choline, betaine, urea and guanine do not interfere. Methylamines and ethylamines give some colour. Lecithin interferes but this interference can be eliminated. The higher amines produced during protein putrefaction also give the test.

The method agrees with distillation methods on fish products and has been found satisfactory.

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Amines in Fish Muscle

II. Development of Trimethylamine and Other Amines

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ABSTRACT

Relatively large amounts of nonvolatile higher amines are formed during proteolysis of fish muscle by bacteria. This follows closely the formation of trimethylamine. Fresh fish contain 0.2 mg. nitrogen per 100 g. muscle as trimethylamine and about 0.1 mg. nitrogen per 100 g. as dimethylamine. Trichloracetic acid and formaldehyde solutions completely extracted trimethylamine from fish and satisfactory recovery of added trimethylamine was obtained.

The simple, rapid measurement of fish quality has always been one of the vexing problems of the fishing industry. Surface pH determination provides a rapid index for stored fillets, but is not satisfactory for round fish or for fillets immediately after cutting, since the surface pH depends on the accumulation of the products of bacterial action in the exposed surface layer, and the test thus measures only the spoilage which has taken place after filleting (Dyer, Sigurdsson and Wood 1944). The trimethylamine level provides the best indication of bacterial deterioration in sea fish of any of the various spoilage effects investigated to date. The studies of Beatty and others (Beatty and Gibbons 1937, Beatty and Collins 1939, Watson 1939) have shown that the bacteria first utilize the lactic acid and sugar in the tissues, simultaneously reducing the trimethylamine oxide present to the odoriferous volatile base trimethylamine. This occurs at first only on the surface tissue, the locus of bacterial growth, so that in an average sample of muscle, the rise in trimethylamine is gradual (Wood, Sigurdsson and Dyer 1942). Thus trimethylamine production constitutes a warning of incipient spoilage by bacterial action.

Dimethylamine and monomethylamine are also formed in small amounts by bacterial action (Beatty and Collins 1940). Shewan (1938) has proposed the measurement of dimethylamine as a spoilage test.

Histamine, phenylethylamine, tyramine, indole and other amines are formed during protein putrefaction, and Geiger (1944) has recently proposed a spoilage test based on the determination of histamine.

The development of the colorimetric method of estimation of trimethylamine (Dyer 1945) resulted in a study of the amines developed over the spoilage curve, a comparison of various extraction methods, and the establishment of the trimethylamine content of fresh fish.

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In Cod Muscle Brei

A suspension of minced fresh cod muscle in an equal weight of distilled water was incubated at 20°C. Samples were withdrawn at intervals for analysis. The proteins were precipitated by the addition of 10 ml. water and 20 ml. of 10 per cent trichloracetic acid to the 10 ml. of muscle brei. Trimethylamine was determined by both the picric acid colorimetric method and the Conway procedure (Beatty and Gibbons 1937). The results are shown in table I.

Table I. Amine formation in cod muscle brei at 20°C.

Incubation time (hours)	Trimethylamine content (mg. nitrogen per 100 g. muscle)		
	Colorimetric	Conway	Difference
12	3.0	4.0	..
18	11.0	10.2	..
20	18	18	..
22	33	31	..
23	38	39	..
25	60	62	..
26	80*	75	5
27	90	77	13
38	125	80	45
45	155	80	75
50	165	80	85
64	210	83	127
86	250	87	163
94	300	95	205
110	350	110	240

*First putrid odour.

The reduction of trimethylamine oxide by the bacterial enzymes follows a logarithmic curve as shown in figure 1.

Thus the reaction is of the first order, from the slow initial stages right through to the completion of the reduction. The results of the two methods of trimethylamine determination agree very well up to the point where the trimethylamine production is almost complete, a level of about 60 mg. nitrogen per 100 g. tissue in the sample under examination. At 26 hours incubation time, the Conway value has reached 75 while the colorimetric method gives 80 mg. nitrogen per 100 g. fish. Hereafter the difference increases at a very rapid rate, the colorimetric value being greater by 240 mg. per 100 g. after 110 hours, as shown in the table. The point of first production of these nonvolatile amines corresponds with the first appearance of the odours associated with protein putrefaction. Many amines are formed during bacterial breakdown of protein, chiefly due to decarboxylation, as discussed more fully by Schmidt (1938, p. 236), and Gale (1940). Putrescine, cadaverine, tyramine, phenylethylamine, and histamine are examples

of those formed. Some of these have been shown to react with the picric acid reagent but they are not sufficiently volatile to be included in the distillation method (Dyer 1945). Thus the production of trimethylamine is closely followed by decomposition or putrefaction of the protein.

This confirms the conclusion reached by Beatty and Collins (1939), who demonstrated the deamination of amino acids in cod muscle press juice during advanced spoilage and who suggested the possible formation of amines by the decarboxylation of amino acids.

In Stored Cod Muscle

Very similar results were obtained on cod muscle. Fresh cod fillets, two or three hours out of the water, were stored at 2°C. Samples were removed at intervals and extracted with trichloracetic acid, a 25 g. minced sample being shaken

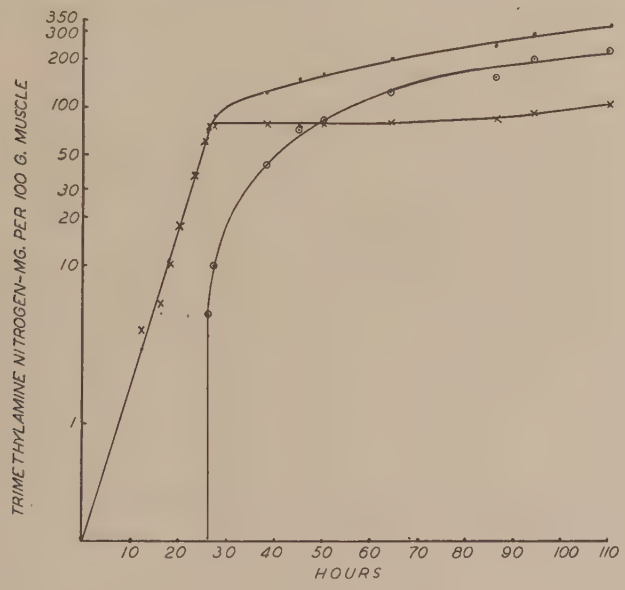

Figure 1. Amine production in cod muscle brei.

with 25 ml. water and 50 ml. of 10 per cent trichloracetic acid. The filtered extract was analysed by the colorimetric and Conway methods in triplicate, with results as shown in table II.

The two methods are in excellent agreement, and development of higher amines did not occur until the fish was quite putrid. Results from the analysis of a large number of samples of spoiled fish showed that no significant development of these amines occurred until the fish had reached a trimethylamine content of about 60 to 80 mg. nitrogen per 100 g. muscle. Organoleptically, cod and haddock muscle is considered undesirable if the trimethylamine content is over 10 or 20 mg. nitrogen per 100 g. tissue so that the measurement of these amines is of no value as an indicator of spoilage, and they do not affect the results obtained by the colorimetric method over the useful range.

Since the production of these amines occurs only during protein breakdown, when the product has reached an advanced state of decomposition, the content of these nonvolatile amines might be a useful indication of protein putrefaction in meats as well as in fish. This is of particular significance since many of the amines produced are quite poisonous, exhibiting marked sympathomimetic activity.

The development of trimethylamine in fish muscle is not logarithmic, since the bacteria act only on the surface and a composite sample is analysed. No development of higher amines was detected at the lower values even though some protein decomposition would be expected in the highly contaminated surface area. Analysis of surface samples would possibly show such a reaction.

TABLE II. Development of amines in cod muscle stored at 2°C.

Time of storage (days)	Trimethylamine content (mg. nitrogen per 100 g. muscle)			
	Colorimetric		Conway	
0	0.19	(± 0.02)	0.23	(± 0.04)
	0.22	(± 0.07)	0.22	(± 0.06)
2	0.36	(± 0.05)	0.30	(± 0.1)
3	0.33	(± 0.14)	0.40	(± 0.2)
4	0.31	(± 0.07)	0.34	(± 0.1)
5	0.81	(± 0.08)	0.80	(± 0.1)
6	2.7	(± 0.2)	2.7	(± 0.1)
7	32.5	(± 4)	31.5	(± 3)
12	119.	(± 3)	94.	(± 1)

Samples of fillets, smoked fish and salt fish are not infrequently found on the market with trimethylamine values of 60 mg. nitrogen or over although only rare cases of food poisoning from decomposed fish are encountered. The presence of the easily recognized trimethylamine base should serve as a warning of incipient protein putrefaction.

TRIMETHYLAMINE AND DIMETHYLAMINE IN FRESH FISH

In the above experiment and in numerous other analyses of fresh cod and haddock muscle made in the laboratory, fresh muscle was found to contain approximately 0.18 to 0.20 mg. nitrogen per 100 g. muscle as trimethylamine. Fish only a few hours out of the water gave the same value as those stored for longer periods but in which trimethylamine oxide reduction had not begun. Thus the enzymic equilibrium in sterile fish muscle tissue greatly favours the oxide form, the ratio of trimethylamine oxide to trimethylamine being about 100 to 0.2.

Shewan (1939) suggests that the apparent trimethylamine obtained by the use of the Beatty and Gibbons' method on fresh cod during the initial stages of bacterial breakdown is actually dimethylamine. Dimethylamine gives only 20 per cent as much colour as trimethylamine in the colorimetric method (Dyer 1945). Thus the close agreement between the colorimetric and distillation

methods is evidence that very little dimethylamine is formed since it is determined in the distillation method. Using the method given below, fresh cod was found to contain about 0.1 mg. dimethylamine nitrogen per 100 g. muscle. Thus the colorimetric procedure would show 0.02 mg. nitrogen per 100 g. muscle as trimethylamine due to the dimethylamine present or only 10 per cent of the trimethylamine found. A similar relation holds for the early part of the spoilage curve.

Dimethylamine Production in Cod Muscle

Dimethylamine was first determined in fish by Reay (1938) and later by Shewan (1938, 1939) and Beatty and Collins (1940) who used the method of Dowden (1938) for dimethylamine in urine. We experienced many difficulties in the application of the method to fish tissue and a study of the reaction was made using the photo-electric colorimeter. The method adopted was as follows. An aliquot of sample solution or trichloracetic acid extract of fish containing 0.001 to 0.006 mg. dimethylamine nitrogen is diluted to 10 ml., 1 ml. of copper-ammonia reagent (20 g. ammonium acetate and 0.2 g. copper sulphate dissolved in 30 ml. water added to a solution of 10 g. sodium hydroxide in 25 ml. water and 20 ml. concentrated ammonium hydroxide all diluted to 100 ml.) is added, followed by 10 ml. of 5 per cent solution of carbon disulphide in benzene. The mixture is heated in a water bath at 40 to 50°C. for 5 minutes. The tubes are tightly stoppered and shaken rapidly in a shaking machine for 5 minutes. One ml. of 30 per cent acetic acid is added and shaken until the solution is clear, about 10 or 20 times. The benzene layer is decanted, dried by shaking with about 0.4 g. anhydrous sodium sulphate and the yellow colour of copper dimethyldithiocarbamate measured in the photoelectric colorimeter using a filter with maximum transmission at 4400 ångström units. The blank is made with distilled water instead of the sample solution. The colour is stable and is proportional to the concentration within the limits given as shown in figure 2. Complete recovery of dimethylamine added to fish extracts was obtained.

Results on fresh cod and haddock gave values of about 0.1 mg. nitrogen per 100 g. This increases to about 3 or 4 with spoiled fish. The development of dimethylamine occurs early in the spoilage cycle but the values were found to be too variable for the comparison of different lots of fish. Thus it is not recommended as a spoilage test.

Trimethylamine as a Spoilage Indicator

Beatty (1938) has shown that at least 94 per cent of the trimethylamine in spoiling fish originates from the reduction of trimethylamine oxide. Keeping (unpub.) and Wood and Keeping (1944) have shown that a few species of bacteria, certain of which may be found on fish, can produce trimethylamine from choline in a culture medium, but only in the absence of an easily oxidizable carbohydrate source. Thus the lactic acid present in fish muscle would probably suppress the reaction. Furthermore, only a very small proportion of the bacteria contaminating fish muscle can split choline, and only traces of trimethylamine are found in fresh water fish (Lintzel, Pfeiffer and Zippel 1939, Shewan 1942).

Fletcher, Best and Solandt (1935) have shown that cod muscle contains only the equivalent of 9 mg. choline nitrogen per 100 g., so that trimethylamine production from choline is not likely to be important, and would in any case be a bacterial spoilage product.

Working with Pacific coast fish, Tarr (1940) has suggested that spoilage in fish containing trimethylamine oxide may occur without trimethylamine formation. As far as Atlantic ground fish are concerned, this is only a rare occurrence; out of more than 3,000 samples examined in the past year only one case was found where the trimethylamine was low and protein degradation was evident. Results obtained in this laboratory (F. E. Dyer unpublished) have shown that from 10 to 40 per cent of the organisms present in fish slime and spoiling fish

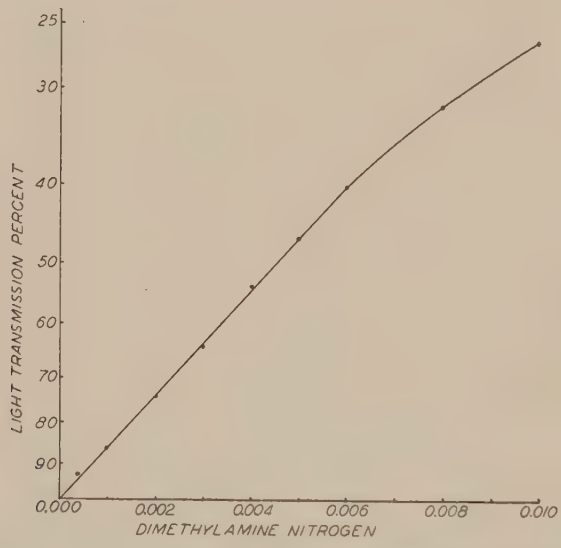

FIGURE 2. Standard curve for dimethylamine. Per cent light transmission, logarithmic scale, plotted against mg. dimethylamine nitrogen per tube.

reduce trimethylamine oxide and that these are active in the presence of other forms.

EXTRACTION METHODS

Previous methods of trimethylamine determination have used weighed samples of fish directly, or the press juice obtained by pressing out the sample. Because of its greater convenience an extraction procedure has been developed. Trichloracetic acid extracts have been used, usually on 10 and 20 g. minced samples. The sample is mixed with an equal weight of water, followed by the addition of a 10 per cent solution of trichloracetic acid, in amount twice that of the water added. The mixture is shaken several times over a period of a half hour or longer and is then filtered, although often an aliquot is removed from the supernatant liquid for analysis without filtration. For the inspection of large numbers of samples of frozen fish for the British Ministry of Food, a method was

developed for taking samples for shipment to the laboratory for analysis. Pint jars containing 50 ml. of a formaldehyde solution, commercial formalin diluted with an equal volume of water, were used, and an approximately 100 g. sample of the fillet to be tested was placed in the jar, which was then sealed. The mixture of sample and formaldehyde solution was weighed at the laboratory to determine the actual sample weight, 150 ml. of water was added, and the mixture pulped in the Waring Blendor. After standing a few minutes the supernatant liquid was decanted and used for the trimethylamine determination. Since the colorimetric method is more sensitive than the microdistillation technique, the use of greater dilutions is permissible than with the latter. Both the formaldehyde and trichloracetic acid extraction methods in conjunction with the colorimetric tri-

TABLE III. Comparison of extraction methods.

Method of extraction	Trimethylamine nitrogen found		
	Mg. nitrogen (av. per 100 g. muscle)		Percentage of distillation method
Distillation*—2 g. fish	12.0 12.0 12.0	12.0	100
Trichloracetic acid 20 g. fish + 20 ml. water + 40 ml. trichloracetic acid	11.5 11.0 11.6	11.4	95
10 g. fish + 30 ml. water + 40 ml. trichloracetic acid	11.5 11.1 11.5	11.4	95
Formaldehyde. 100 g. fish + 50 ml. formaldehyde + 150 ml. water	12.0 11.2 12.0	11.7	97.5
50 g. fish + 50 ml. formaldehyde + 150 ml. water	11.9 11.0 11.9	11.6	97

*Beatty and Gibbons semimacrodistillation.

methylamine determination have been used successfully on many thousands of samples over the past two years. In table III are shown the results of an experiment in which the above procedures are compared with the Beatty and Gibbons distillation method on the same well comminuted sample of cod muscle.

The three methods are in good agreement, and although the results obtained by the trichloracetic acid extraction were not quite as high, 95 per cent of those by the direct distillation method, this is quite satisfactory for practical use in a spoilage test. Stansby et al. (1944) obtained very poor extraction of trimethylamine from fish, using trichloracetic acid. These authors used press juice with a small volume of concentrated trichloracetic acid solution added, and found that

considerable trimethylamine remained entrained in the voluminous protein precipitate. In the present work, 1 to 4 and 1 to 8 dilutions of the original muscle were used with satisfactory results.

Known amounts of trimethylamine added to fish muscle were recovered satisfactorily by all the above methods as shown in the experiment reported in table IV.

TABLE IV. Recovery of trimethylamine in three extraction methods.

Method of extraction	Trimethylamine nitrogen found (mg. N per ml. extract)				Trimethylamine recovered	
			Av.	Added	mg. nitrogen per ml.	Per cent
Distillation—2 g. fish	0.24	0.24	0.24 — 0.24			
" + 1 mg. N-Trimethylamine	1.22	1.22	1.22 — 1.22	1.00	0.98	98.0
Trichloracetic acid						
20 g. fish + 20 ml. water						
" + 40 ml. acid	0.0278	0.0272	— 0.0275			
" + 2 mg. N-Trimethylamine	0.524	0.528	— 0.526	0.025	0.0251	100.0
10 g. fish + 30 ml. water						
" + 40 ml. acid	0.0132	0.0147	— 0.0140			
" + 2 mg. N-Trimethylamine	0.0390	0.0400	— 0.0395	0.025	0.0255	102.0
Formaldehyde						
100 g. fish + 50 ml. formaldehyde						
" + 150 ml. water	0.038	0.042	— 0.040			
" + 10 mg. N-Trimethylamine	0.076	0.074	— 0.075	0.0333	0.035	105.0
50 g. fish + 50 ml. formaldehyde						
" + 150 ml. water	0.0206	0.0226	— 0.0216			
" + 5 mg. N-Trimethylamine	0.0420	0.0400	— 0.410	0.0200	0.0194	97.0

The formaldehyde extract still contains considerable protein solution but this does not affect the results obtained by the colorimetric method. Similarly, direct determinations on the pickle from canned fish without protein precipitation also show no interference.

From the above results it is concluded that trimethylamine in fish muscle may be extracted by trichloracetic acid or formaldehyde solution and the trimethylamine in the extract determined quantitatively by the picric acid colorimetric method.

SUMMARY

In the bacterial spoilage of a cod muscle brei trimethylamine is first produced followed by large amounts of higher non-volatile amines. Similar results were obtained on cod muscle. This occurred only in the advanced stages of deterioration of the muscle, at trimethylamine values of about 60 to 80 mg. nitrogen per 100 g. muscle or over. Thus food poisoning from these amines, many of which are highly active physiologically, is possible if badly spoiled fish muscle is ingested.

Fresh cod and haddock contain about 0.2 mg. nitrogen per 100 g. muscle as trimethylamine. This is not dimethylamine as has been suggested, the dimethylamine content of fresh cod and haddock muscle being about 0.1 mg. nitrogen per 100 g. Dowden's method for the determination of dimethylamine has been studied and modified for application to fish muscle extracts.

Extraction of fish muscle with trichloracetic acid and formaldehyde solutions gave practically complete extraction of the trimethylamine present as determined by distillation methods. Satisfactory recovery of trimethylamine added to fish muscle was also obtained with each of the three methods, distillation and trichloracetic acid and formaldehyde extraction.

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Factors Affecting Triamineoxidease
I. Inhibition of the Enzyme

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ABSTRACT

Ammonium hydroxide and glycogen have little effect on the formation of trimethylamine from trimethylamine oxide by washed cells of bacteria isolated from fish. Indole, skatole and hydrogen sulphide partially inhibit the enzyme. Partial inhibition can also be effected with cyanide and azide. The necessity in this system of a hydrogen carrier other than the dehydrogenase activating the oxidizable substrate is suggested.

Suwa (1909) was the first to show that the trimethylamine oxide now known to be common to nearly all sea fish (Beatty 1939) is reduced to the corresponding amine in the course of bacterial action on the muscle. This discovery subsequently led to the observation that autolysis plays a minor role in volatile base formation (Beatty and Gibbons 1937) and that the specific bacterial species possessing the enzyme triamineoxidease (Watson 1939a, 1939b; Tarr 1939, 1940, 1941) use this respiratory catalyst in a system where trimethylamine oxide is activated as a hydrogen acceptor (Quastel, Stephenson and Whetham 1925).

Recent investigations (Tarr and Sunderland 1938, Tarr and Bailey 1939) have questioned the value of trimethylamine determination as an adequate method for detection of sea fish spoilage. However Baird (unpub.) has examined a number of representative species of different genera listed in Bergey's (1939) Manual and has demonstrated that the power to reduce trimethylamine oxide is fairly widely distributed, especially in the family Enterobacteriaceae (Wood and Baird 1943).

Since very little is known concerning the factors affecting trimethylamine formation by bacteria, a study of some of these factors has been undertaken, of which the present paper is intended to represent an initial communication.

EXPERIMENTAL

Cultures

A sample of cod muscle weighing 1 g. (trimethylamine nitrogen value 2.5 mg. per cent) was finely shredded in a 100 ml. dilution blank containing broken glass and the resulting suspension plated quantitatively to nutrient agar. From the low dilution plates a total of 55 organisms were isolated, 5 of which (numbers 3, 6, 37, 45 and 53) were proven to be trimethylamine positive (Wood, Baird and

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Keeping 1943). The latter unfortunately do not show sufficiently fixed characteristics to permit definite classification, but cultures of them are being maintained for whatever use may be desirable.

Technique

Unless otherwise stated, washed cells (Sandiford and Wooldridge 1931) grown on nutrient agar and centrifuged twice (Guggenheim 1944) from physiological saline were used throughout. The virtues of this technique are reviewed in detail by Wilson (1938). If the freshly prepared suspensions could not be used immediately, they were stored at 0°C., the dry weight per ml. of cell suspension being recorded in each case.

Observing strict aseptic technique, Thunberg (1918) vacuum tubes charged with the following were used to carry out the reduction: 1 ml. cell suspension; 1 ml. 0.1 M trimethylamine oxide; 1 ml. 0.1 M oxidizable substrate (sodium acetate, glucose, etc.); 1 ml. phosphate buffer pH 7.0; solution of inhibitor or water to given volume.

The trimethylamine formed was determined after incubating for 10 hours at 37°C. by the colorimetric method of Dyer (1945). Of a number of compounds tested, sodium acetate was found to stimulate reduction in the case of each positive culture. Proof that the yellow colour obtained was the picrate of trimethylamine, and not that of higher amines or substances introduced with the cell suspension, was determined by incubating each culture in tubes where water replaced the oxide, the Evelyn photoelectric colorimeter giving a zero reading for every culture tried. In practice it was found unnecessary to prepare Thunberg tubes in duplicate, although two trimethylamine determinations were always carried out, and the figure shown is the average of the two values. The discrepancy for as little as 2.5 micrograms trimethylamine nitrogen was never more than 8 per cent.

Effect of Ammonium Hydroxide

Not many workers have reported the effect of ammonia on bacterial enzymes. A generous amount of substrate exists in the muscle of all fish for the amino acid

Table I. Effect of ammonium hydroxide on triamineoxidase

Ammonium hydroxide (%)	Organism number				
	3	6	37	45	53
	Trimethylamine nitrogen (γ per 0.5 ml. culture)				
0.000	33.5	32.1	26.2	43.5	25.4
0.011	32.1	29.4	22.7	43.6	24.1
0.023	30.8	30.8	26.8	42.1	22.7
0.118	29.4	32.1	33.5	41.3	21.4
0.236	34.8	37.5	28.1	42.1	25.4
Dry wt. (mg. per exp.)	14.2	17.5	12.2	18.8	11.0

oxidase; while certain other species (families Squalidae and Rajidae) have muscle containing much urea.

Ammonia was added as 1.42 per cent ammonium hydroxide and the phosphate buffer was increased to 2.0 ml. making a total volume of six ml. The results (table I) show that ammonium ions have little effect on triamineoxidease.

Effect of Glycogen

Watson (1939b) has stated that there exists in fish muscle a sufficient amount of carbohydrates and their derivatives to reduce all the trimethylamine oxide present. Pyruvate, lactate, glucose, and to a lesser extent glycogen, were all activated by his so-called "reducing *Achromobacter*" as hydrogen donors. Because of the very wide variety of molecules capable of arising from glycogen degradation we have tested its influence in the case of our cultures. Table II shows that when glycogen replaces sodium acetate as a source of hydrogen, very little oxide is reduced.

Table II. Effect of glycogen on triamineoxidease

Glycogen (*M*)	Organism number				
	3	6	37	45	53
	Trimethylamine nitrogen (γ per 0.5 ml. culture)				
0.0000	3.2	3.2	3.0	4.2	3.9
0.0002	3.9	3.7	2.0	3.3	4.3
0.0020	3.0	4.5	4.2	4.2	4.9
0.0200	2.5	2.8	3.9	3.6	4.4
Dry wt. (mg. per exp.)	13.7	14.7	16.8	12.0	15.1

Effect of Indole and Skatole

The investigations of Gordon and McLeod (1926) and France (1936) show that concentrations of indole of the order of 1:1500 completely inhibit growth of certain bacteria.

Table III. Effect of indole (A) and skatole (B) on triamineoxidease.

Organism number	Indole or skatole (concentr.)								Dry wt. (mg. per exp.)	
	0:0		1:40,000		1:4,000		1:800			
	Trimethylamine nitrogen (γ per 0.5 ml. culture)									
	A	B	A	B	A	B	A	B	A	B
3	25.0	26.7	27.5	25.3	10.0	12.2	3.7	1.8	11.7	11.7
6	30.7	24.0	29.4	23.7	14.3	13.7	6.6	2.8	12.7	14.2
37	25.6	26.0	23.1	25.3	9.8	14.0	3.6	4.0	12.6	15.3
45	20.1	19.2	19.2	28.2	3.5	13.2	0.7	3.0	12.6	11.0
53	25.3	26.7	16.1	26.3	3.7	12.6	0.7	2.2	10.0	13.5

The tryptophane fraction of fish muscle protein is probably close to 0.95 per cent (Bailey 1937). Hence considerable amounts of the benzene-pyrrole derivatives of this amino acid could be formed under optimum conditions.

Skatole was dissolved in 5 per cent ethyl alcohol, the control in this case receiving 1 ml. of 5 per cent ethyl alcohol instead of water.

The figures in table III suggest that a concentration of 1:4000 indole or skatole has some inhibitory effect on trimethylamine formation.

Effect of Hydrogen Sulphide

The sulphur content of cod muscle calculated to be in the neighbourhood of 250 mg. per cent (wet weight) (McCance and Widdowson 1940, p. 47) could under extreme bacterial proteolysis be converted completely to hydrogen sulphide. Since sulphides have been known to stabilize the metallo groups of res-

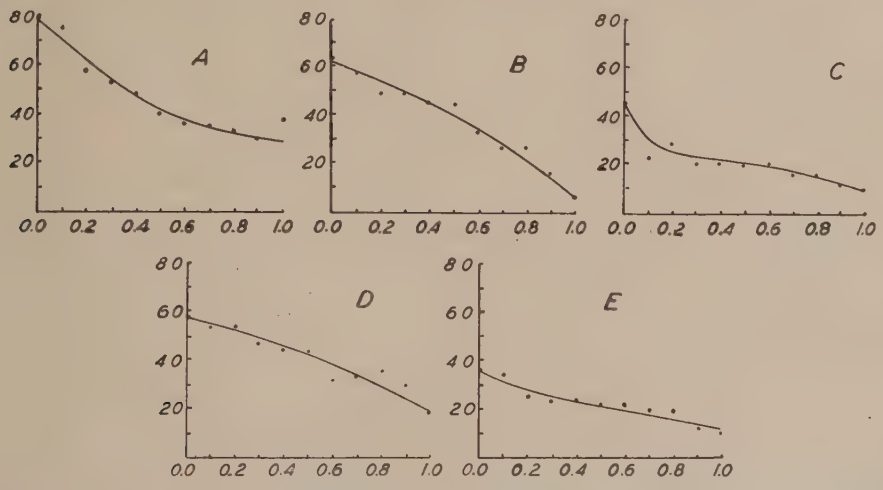

Figure 1. The inhibition of the triamineoxidease activity of washed cells of five bacterial cultures by hydrogen sulphide. Abscissae,—ml. saturated H_2S solution. Ordinates,—γ trimethylamine nitrogen per 0.5 ml. culture.

piratory enzymes (Elvehjem, Wilson, *et al.* 1939; Sumner and Somers 1943) the effect of this inhibitor has been tested.

Sulphide was added from a saturated solution of the gas. Figure 1A, B, C, D, and E suggests a direct stoi chemical relation between sulphide concentration and enzyme inhibition.

Pre-treatment

In order to determine whether or not this damage persisted in cells pretreated with hydrogen sulphide and washed free from the poison the following experiment was undertaken.

One ml. amounts of a cell suspension were placed in small conical centrifuge tubes. Observing the usual strict aseptic precautions, saturated hydrogen sulphide solution, 1 to 3 ml. was added to half of one series of tubes, and the contents

of each tube of the series were made up to 7 ml. with water. At the end of one hour at room temperature, the cells were deposited by strong centrifugation, washed once and taken up in 2 ml. of saline. Thunberg tubes were prepared as before with water to a total volume of 7.0 ml.

The results shown in table IV indicate that the hydrogen sulphide has imposed a permanent injury upon the cell.

TABLE IV. Effect of hydrogen sulphide on triamineoxidease.

Treatment	Organism number				
	3	6	37	45	53
	Trimethylamine nitrogen (γ per 0.5 ml. culture)				
Control	9.4	13.1	11.8	13.9	10.2
Cells pre-treated with 1.0 ml. sat. H_2S solution	7.7	11.5	11.8	9.3	10.2
Cells pre-treated with 3.0 ml. sat. H_2S solution	2.1	9.4	4.3	4.2	4.6
Cells in contact with 1.0 ml. sat. H_2S solution	8.0	8.2	2.5	7.2	10.2
Cells in contact with 3.0 ml. sat. H_2S solution	6.1	10.2	2.5	4.2	7.5
Dry wt. (mg. per exp.)	5.6	12.4	9.0	11.6	9.5

EFFECT ON DEHYDROGENASES

Sulphides, like cyanides, are generally believed to be without effect on cell dehydrogenases (Elvehjem, Wilson, *et al.* 1939; Green 1940). The influence of hydrogen sulphide on the dehydrogenases of our positive cultures was determined as follows.

A heavy suspension of washed cells was made up to 10 ml. with saline, and divided. To one-half (control) in a small Erlenmeyer was added 30 ml. of water while the other half in a second flask received 10 ml. of a saturated hydrogen sulphide solution and 20 ml. water. The flasks were held for one hour at room temperature, at the end of which time their contents were centrifuged, washed and suspended in 10 ml. of saline.

The separate suspensions were then tested by the Thunberg (1918) technique for ability to reduce methylene blue in the presence of a variety of organic substrates. The tubes received 1 ml. of 0.1 M substrate, 1 ml. of cell suspension added via the hollow stopper subsequent to evacuation, 1 ml. phosphate buffer pH 7.0 and 1 ml. of 1:5000 methylene blue. The tubes were evacuated simultaneously with the same water pump and incubated in water at 38°C. for one hour.

Only in certain instances (table V) did sulphide treatment impair dehydrogenase activity, and then the effect seemed to be one of retardation rather than

inhibition. It is obvious that the effect of sulphide on cell dehydrogenases varies with the substrate and cultures employed. However proof is presented that the cell does have dehydrogenases not affected by hydrogen sulphide.

Whether or not these systems are active when trimethylamine oxide replaces methylene blue as hydrogen acceptor is of course a matter of speculation.

TABLE V. Effect of hydrogen sulphide on cell dehydrogenases. (Conditions given in text)

Substrate	Organism number									
	3		6		37		45		53	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Sodium lactate .			++++	++++	+++	++	+++	+++	++++	++
Sodium formate.		++	+	+			+	++++	+	+++
Sodium acetate.	++++	++++	+	+	+	++++	++++	++++	+	++
Sodium citrate .	++++	++++	++	++	+	+++		+++		
Maltose.		++++				+++	+	+++		+
Sucrose .	+++	++						+++	++	++++
Fructose.	++++	++++				++	+	+++	++	++++
Dry wt. (mg. per exp.) . . .	7.7	7.8	13.2	13.7	4.0	2.9	3.3	4.0	9.8	6.1

EFFECT OF CYSTEINE AND SODIUM THIOGLYCOLLATE

If the mechanism of sulphide inhibition is intimately linked with the strong reducing character of this substance, it should be possible to demonstrate a similar inhibition with cysteine and thioglycollate. Table VI shows the effect of

TABLE VI. Effect of sodium thioglycollate (A) and cysteine hydrochloride (B) on triamineoxidease

Concentr. (*M*)	Organism number									
	3		6		37		45		53	
	Trimethylamine nitrogen (γ per 0.5 ml.)									
	A	B	A	B	A	B	A	B	A	B
0.0000	21.7	10.5	31.6	30.7	33.9	27.5	37.6	33.0	37.3	18.2
0.0002	24.0	10.9	36.5	26.5	33.0	21.3	37.3	35.5	32.2	17.9
0.0020	22.3	7.7	33.4	22.1	36.8	16.5	36.0	29.0	38.5	14.5
0.0200	25.3	6.9	33.6	16.0	36.8	13.7	38.8	13.9	32.2	8.8
Dry wt.. (mg. per exp.)	14.2	13.7	13.1	14.7	14.3	16.8	16.5	12.0	17.5	15.1

adding different concentrations to these compounds. The inhibition brought about by cysteine may be connected with its ease of oxidation from the –SH– to the –SS– form. Also, in spite of the extreme reducing character of the medium, the intracellular mechanism of the cell may remain very well poised.

Effect of Cyanide and Azide

By employing the inhibitors cyanide and azide the results obtained with hydrogen sulphide were confirmed as inhibition through stabilization of the metallo groups of the oxidase enzymes (tables VII and VIII). These substances were introduced as 0.1 M solutions of potassium cyanide and sodium azide after all other reagents had been added including sufficient water to make a final volume of 5.0 ml. Cyanide does not interfere with Dyer's colorimetric test.

Table VII. Effect of cyanide on triamineoxidease.

Organism number	Potassium cyanide (*M*)				Dry wt. (mg. per exp.)
	0.000	0.002	0.004	0.008	
	Trimethylamine nitrogen (γ per 0.5 ml.)				
	After 10 min. incubation				
3	30.2	12.9	2.4	0.0	14.2
6	44.2	33.9	23.0	22.1	13.1
37	44.2	33.9	23.1	16.9	14.3
45	43.7	33.6	21.2	13.6	16.5
53	41.7	30.1	21.7	13.6	17.5
	After 50 min. incubation				
3	50.1	46.2	32.0	26.9	9.2
6	45.7	30.7	26.7	19.1	9.7
37	40.0	28.4	20.1	14.8	15.2
45	39.4	28.2	25.5	15.3	11.1
53	38.2	24.4	19.7	14.1	16.3

Table VIII. Effect of azide on triamineoxidease.

Organism number	Sodium azide (*M*)				Dry wt. (mg. per exp.)
	0.000	0.002	0.004	0.008	
	Trimethylamine nitrogen (γ per 0.5 ml.)				
3	40.3	38.2	35.4	32.0	11.7
6	37.1	34.1	31.8	20.5	12.7
37	37.1	36.2	36.0	22.1	12.6
45	19.6	10.3	9.1	7.1	12.6
53	14.2	8.7	6.4	3.0	10.0

Certain investigators (Bernheim 1942, pp. 30-31) working with other enzymes have reported inhibitions by much smaller concentrations of cyanide when steps were taken to prevent loss of the poison through distillation, and by using a bicarbonate instead of a phosphate buffer. Figure 2 shows the rate of reduction by washed cells of organism no. 37 in the presence and absence of cyanide when the incubation was carried through to the usual ten hours. The inhibition noted at the end of 50 minutes' incubation is given in table VII. These results strongly suggest that cyanide reacts only with a fraction of the enzyme, allowing the remainder to become "saturated" with the substrate in the sense of Stotz *et al.* (1938).

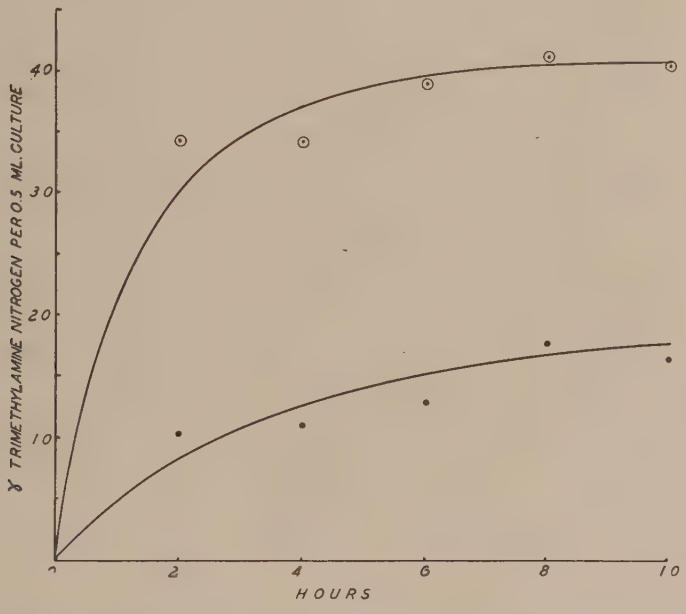

FIGURE 2. The effect of cyanide on the rate of triamineoxidease activity. 0————0 Organism no. 37 with H_2O (control). .————. Organism no. 37 with 0.008 *M* KCN.

A confirmation of the above result was obtained in the following way:

Thunberg tubes were prepared containing 1.0 ml. of 0.1 M sodium acetate, 1.0 ml. of 0.2 M phosphate buffer pH 7.0, 1.0 ml. of 1:5000 methylene blue, 1.0 ml. cell suspension and 0.1 M potassium cyanide or water to 5.0 ml. The hollow stoppers were charged with 1.0 ml. of 0.1 M trimethylamine oxide. The tubes were then evacuated and incubated in water at 37°C. until the dye had been completely reduced. The oxide was then run in, causing in each case a very slight re-oxidation of the dye. In some cases, however, the dye was again rapidly reduced and this observation has been recorded as "no change." Results are expressed in table IX.

In the absence of cyanide, the re-oxidation of the dye proceeded rapidly to completion.

Where the cell is furnished with leuco methylene blue as a source of hydrogen, a specific dehydrogenase enzyme may be considered to be unnecessary, the dye

being perfectly autoxidizable. But it is clear from the results recorded in table IX that in the case of cyanide poisoning the ability of the cells to re-oxidize the dye in the presence of trimethylamine oxide is either partially or completely destroyed.

TABLE IX. The ability of triamineoxidease to re-oxidize reduced methylene blue in the presence of cyanide and trimethylamine oxide.

Organism number	KCN (M)	Methylene blue reduction time (min.)	Re-oxidation of $MeBH_2$ 50 min. after adding oxide	Trimethylamine nitrogen (γ per 1.0 ml.) after 50 min.	Dry wt. (mg. per exp.)
3	0.000	5	Complete	21.5	18.1
	0.002	10	Slight	13.3	"
	0.004	5	No change	8.2	"
6	0.000	5	Complete	12.3	13.2
	0.002	10	Slight	6.9	"
	0.004	10	"	6.0	"
37	0.000	5	Complete	12.7	14.1
	0.002	15	No change	10.3	"
	0.004	15	"	3.5	"
45	0.000	10	Complete	12.3	18.8
	0.002	15	Slight	4.2	"
	0.004	20	No change	2.2	"
53	0.000	5	Complete	7.1	10.9
	0.002	15	Slight	3.9	"
	0.004	20	No change	1.7	"

Wieland (1932, p. 37) claims that M/50 hydrocyanic acid is only capable of a 50 per cent inhibition of the velocity of the reaction catalyzed by acetic acid dehydrogenase. It is obvious from our experiments (table VII) that concentrations of cyanide five times smaller than those employed by Wieland exercise an appreciable inhibitory effect on the activity of triamineoxidease.

Effect of Other Cultures

To establish whether or not the introduction of a suspension of a non-trimethylamine oxide-reducing organism measurably affected trimethylamine formation, the following experiment was carried out:

All cultures isolated, totalling 55 in all, were inoculated into 1 g. amounts of ethylene-oxide sterilized fish muscle, Wood and Morgan (unpublished data) suspended in 2.0 ml. distilled water, the hydrogen sulphide elaborated being detected by enclosing a strip of filter paper impregnated with lead acetate in the mouth of the tube. Eleven hydrogen-sulphide-forming cultures, nos. 5, 15, 18, 24, 25, 26, 27, 29, 32, 38 and 47 were obtained.

One ml. suspensions of non-trimethylamine oxide-reducing organisms were then incubated in Thunberg tubes with 1 ml. of an oxide-reducing suspension

employing a Seitz sterilized 1-4 dilution of cod muscle extract as hydrogen donor. To each of the oxide-reducing organisms (nos. 3, 6, 37, 45 and 53) was assigned a group of 10 negative organisms. In no case did the introduction of a non-trimethylamine-forming culture interfere with the activity of an oxide-reducing culture. We conclude that under these conditions the rate of triamineoxidease activity greatly exceeds that of the proteolytic enzymes rendering the otherwise toxic products of the protein-hydrolysing group without effect on the oxide-reducing system.

DISCUSSION

Of the naturally occurring substances tested, indole, skatole and hydrogen sulphide inflict greatest inhibition upon bacterial triamineoxidease. The respiratory inhibitors said to have a parallel action with sulphides, viz. cyanide and azide also inhibit the enzyme.

Since nitrate and trimethylamine oxide are so similar as to the kinds of bacteria reducing them and as to being hydrogen acceptors (Watson 1939a, 1939b; Tarr 1940; Green 1940) it is significant to note certain characteristics of the nitratase. Yamagata (1938), Broh-Kahn and Mirsky (1938), and Stickland (1931) report the nitrate-reducing enzyme to be cyanide sensitive in *B. coli*, while Lichstein and Soule (1944) have recently noticed that azide prevents the anaerobic growth in nutrient broth of a number of aerobic facultative-anaerobic species.

There is little doubt that both oxidizable and reducible components of our system require activation by the bacterial cell. The original views of Warburg (1925) concerning the inhibition by hydrocyanic acid of an iron catalyst in carbon dioxide assimilation, nitrate assimilation and hydrogen peroxide decomposition could conceivably be extended to cover also trimethylamine oxide reduction.

On the other hand, Ball (1943, pp. 16-32) has sought a new way to explain cyanide inhibitions especially where higher concentrations of the poison were used. Ball believes that in muscle, cyanide depresses the redox potential below that of cytochrome *a* or *c* resulting in a maximum inhibition of respiration while azide is only able to drop the potential below that of cytochrome *a* allowing 50 per cent of respiration to proceed through cytochrome *c*. Further, azide and cyanide have no effect on the cytochrome oxidase of the unfertilized arbacia egg since in this case the enzyme reacts directly with a flavin of quite low potential. Fertilization of the eggs by cytochrome rich arbacia sperm initiates cyanide sensitivity.

So far we have been unable to inhibit triamineoxidease with carbon monoxide. We have examined a small number of stock cultures of the family Enterobacteriaceae—all possess a cyanide-sensitive reducing system.

The reduction of trimethylamine oxide therefore appears to require not only an oxidizable substrate with its specific dehydrogenase but also a second carrier or series of carriers between the dehydrogenase and the oxide. Schematically this may be represented:

$$AH_2 \rightarrow A + H_2$$
$$H_2 + X \rightarrow XH_2$$
$$XH_2 + (CH_3)_3N{:}O \rightarrow (CH_3)_3N + H_2O + X$$

where AH_2 is the oxidizable substrate and XH_2 is the reduced form of the cyanide-sensitive carrier.

SUMMARY

Under the conditions employed in these experiments the following results were established:

1. Ammonium hydroxide and glycogen have very little effect on triamine-oxidease.

2. Indole, skatole and hydrogen sulphide partially inhibit the enzyme. Certain specific dehydrogenase enzymes of washed cells of the bacteria used are not affected by hydrogen sulphide treatment.

3. Thioglycollate is without influence on trimethylamine formation although a definite inhibition can be obtained with cysteine.

4. Cyanide and azide cause appreciable inhibition in concentrations smaller than those normally believed to inhibit specific dehydrogenases.

5. The presence of non-reducing organisms including some proteolytic strains, does not measurably affect the reducing activity of a suspension of positive cells when sterile muscle press juice is provided as hydrogen donor.

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Drying of Heavily Salted Fish

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ABSTRACT

The experimental drying of cut samples and of whole salt fish under various air conditions shows that the optimum air velocity is 100 to 125 cm. per second, air temperature 26°C., and relative humidity 45 to 55 per cent. High drying potentials inhibit drying owing to the formation of an impervious salt-protein crust on the surface of the fish.

The skin surface of the fish dries about 50 to 75 per cent as fast as the split surface. Under the recommended air conditions medium size salt codfish are dried from 130 per cent to 75 per cent, dry basis, moisture in about 40 hours.

Until recently, most of the salt fish in the Maritime provinces was dried by exposing it to sun and wind on wooden or wire racks, known as flakes. The difficulties and even serious losses incurred by subjecting this perishable product to the vagaries of the weather, have resulted in numerous efforts to design equipment for drying salt fish by artificial means. An account of the development of early dryers has been given by Stevenson (1898). Several dryers, erected in Halifax, are described by Jeffers (unpublished manuscript). In his review of the Newfoundland salt fish industry, MacPherson (1933) recommended a maximum air temperature of 26.7°C. (80°F.) and indicated that drying may occur at a relative humidity exceeding 70 per cent. Norwegian workers (1934) using a warm air dryer, found the optimum air velocity to be 100 cm. per second (200 feet per minute) and used relative humidities exceeding 75 per cent. Since Hampton (1937) and Cooper (1938) showed that drying cannot occur above 73 per cent relative humidity, the statements of MacPherson and that of the Norwegian investigators may be questioned.

The object of the present investigation is to determine the optimum conditions of temperature, air velocity and relative humidity for the air drying of heavily salted fish. The experimental work has been carried on by various investigators at this laboratory, and on the basis of the data accumulated, artificial dryers have been drying heavily salted fish successfully over a period of approximately five years (Cooper 1942).

The apparatus used for the experimental drying of salt fish has been described by Cooper and Wood (1940). They established the minimum, practicable air flow at approximately 125 cm. per second (250 feet per minute), and reported that a rough undesirable finish is formed by the accumulation of salt crystals on the surface of the fish at lower air velocities.

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Undried salt fish is extremely variable with regard to texture, salt content and water content, the variations arising, in part, from differences in curing procedure and storage temperature. Early experiments showed quite a wide variation in drying rates. To lessen this, and to speed the work, small samples as nearly identical as possible, and cut from typical large salt cod, were used to determine the optimum conditions for drying. This procedure permitted the testing of a large number of individual samples. The data thus obtained were later checked by drying fairly large lots of green or undried fish in a semi-commercial dryer. The raw material was entirely heavily salted fish. Part of this was pickle cured, or stored under saturated sodium chloride solution. Part was kenched, that is salted with an excess of sodium chloride in piles, the water drawn from the fish by the salt flowing away as brine. The water content of the fish, previous to drying, ranged between 55 and 59 per cent and the salt 14 to 18 per cent of the weight of the fish.

CUT SAMPLES OF KENCH CURED COD

Cut samples, 11 cm. long, 4 cm. wide and 7 mm. thick, were taken from the thick part of the kench cured cod. They were weighed on glass plates, then the edges were covered, leaving only the top surface area of about 44 sq. cm. exposed to the air stream. In most experiments, several duplicate samples were dried, one sample always being placed on the recording balance, while the remaining samples were supported on metal screens. The tunnel dryer used provided two regions of different air velocities.

The results of the preliminary experiments indicated that it was not always possible to duplicate results with cut samples. At first, these variations were ascribed to faulty technique in using collodion to prevent drying from the edges of the sample. Paraffin wax, which does prevent evaporation from the edges, was substituted for the collodion, but slight variations still existed. Possibly these discrepancies arose from differences in the fish themselves or from the angle of the cut surface to the muscle fibres. With care in sampling, reasonably good duplication of drying results could be obtained.

Fifteen drying experiments were made, each utilizing several samples of kench cured cod, all at a relative humidity of 50 per cent. Dry-bulb temperatures of 15.6, 21, and 26.7°C. (60, 70, and 80°F.) and air velocities of 100 to 225 cm. per second (200 to 450 feet per minute) were used. Four types of exposed surfaces were studied:

1. A surface trimmed smooth with a razor (fig. 1).
2. The original cut surface from the splitting knife (fig. 2, curve D).
3. The washed skin surface (fig. 2, curve E).
4. The surface of salt fish ground to about one-eighth inch (ca. 3.2 mm.) size and pressed into a cake (fig. 2, curve F).

Various methods for analysing and plotting the data were tried, in an attempt to determine the drying coefficients and the mechanism of the drying of salt fish. The most significant method of plotting the data is shown in figures 1 and 2, in which fractional free water content of the sample (Walker 1937) is plotted against time, on a semi-logarithmic scale. This fractional free water content may be

defined as the ratio of the total water content of the sample less its equilibrium moisture content (Cooper 1938) to the original total water in the sample less its equilibrium moisture, and is, therefore, expressed as $\frac{W}{W_c}$.

Each curve is the mean of several experiments, and falls into two parts, the first part, from the start of the drying period to 550 minutes, being elliptical, and the second, from 550 minutes to the end, being a straight line.

During the first 550 minutes, it seems probable that a parabolic moisture distribution is being set up in the sample and the characteristic hard surface is formed. The straight line portion of each curve indicates that, during this period, drying falls into one of the following classifications:

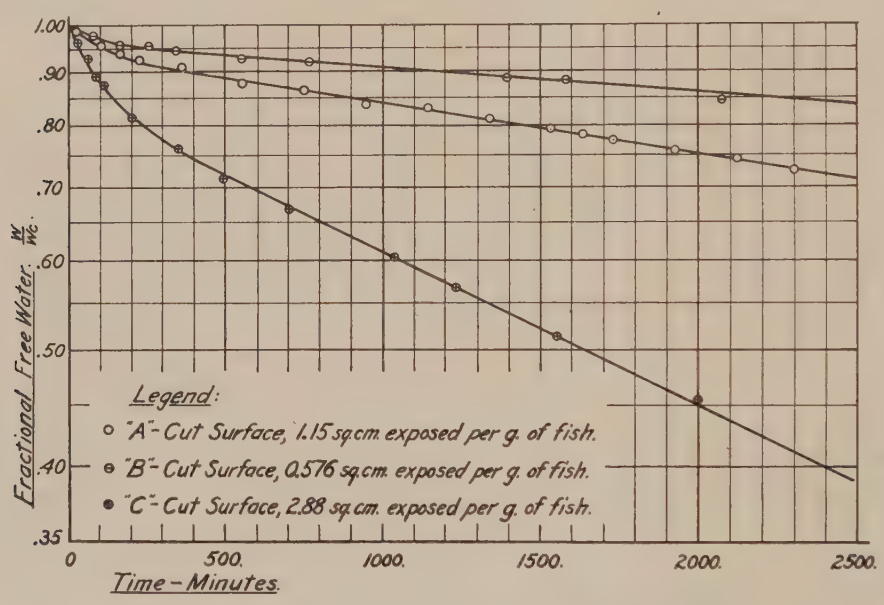

FIGURE 1. Drying curves for heavily salted fish samples.

(*a*) Controlling factor: Resistance to surface evaporation,

$$\log. \frac{\text{(fractional free water)}}{\text{time}} = \frac{\text{(coeff. of surface evaporation)}}{\text{(thickness of sample)}}$$

(*b*) Controlling factor: Resistance to liquid diffusion,

$$\log. \frac{\text{(fractional free water)}}{\text{time}} = \frac{3\text{(diffusivity of water)}}{\text{(thickness of sample)}^2}$$

Consideration of curves *A*, *B*, and *C*, in which the effective thickness of the samples is 7, 14, and 3 mm. respectively, shows that $\log. \frac{\text{(fractional free water)}}{\text{time}}$ varies inversely with the thickness of the sample. Thus, the drying of these samples falls into that classification in which surface evaporation is controlling. Hence, the drying time should be reduced by increasing the air velocity, or decreasing the relative humidity. The interesting fact is that under the air condi-

tions studied, the rate of loss of water is not controlled by diffusion of water from the thick parts of the fish to the surface layer, but presumably it is the thin, dried crust, at the surface of the fish, which inhibits evaporation of water to the surrounding air.

Within the accuracy of the experiments, the drying curves for dry-bulb temperatures of 15.6, 21.1, and 26.7°C. (60, 70, and 80°F.) and air velocities of 100 and 225 cm. per second (200 and 350 feet per minute) were identical with curve *A*. If air velocities much lower than 100 cm. per second (200 feet per minute) are used, an unsatisfactory type of surface is produced.

Curves *A*, *D*, *E*, and *F* illustrate the influence of the type of surface exposed upon the drying rate. The skin (curve *E*) dries about 50 per cent as fast as a

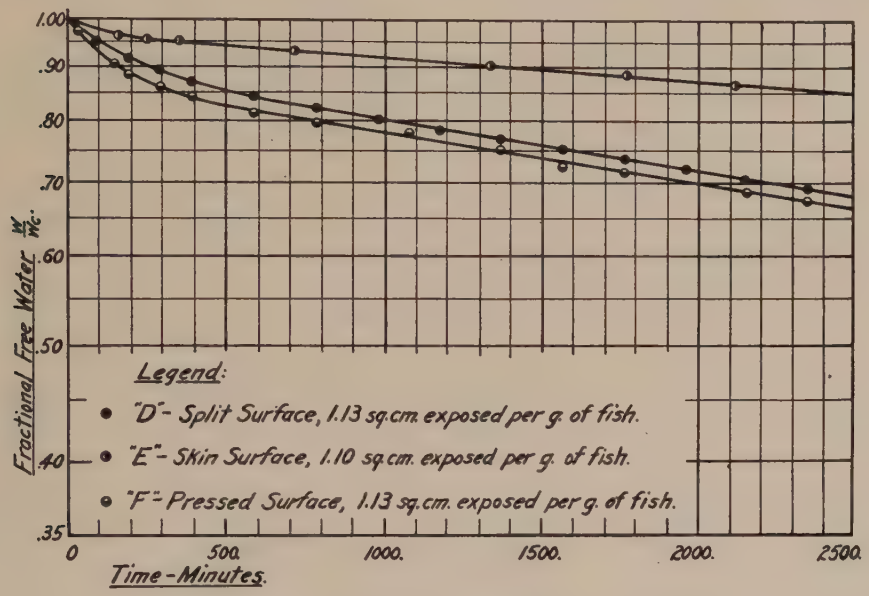

FIGURE 2. Drying curves for heavily salted fish samples.

razor cut surface, (curve *A*) and about 45 per cent as fast as the original split surface of the fish, (curve *D*). The pressed, ground fish (curve *F*) dries considerably faster than other types of surface and obviously does not follow the same type of drying as the cut samples.

CUT SAMPLES OF PICKLED CURED COD

Ten drying experiments, designed to show the effect of variations in dry-bulb temperature, relative humidity and type of surface exposed to the air stream, were carried out, using samples cut from pickle cured cod. The same apparatus and drying technique were used as in the drying of cut samples of kench cured cod. Fresh fish were obtained locally and were salted within a few hours after being caught. They were kept under salt for four to eight weeks. The average water content of the fish was 59 per cent, wet basis (140 per cent, dry basis). The average salt content was 48 per cent, dry basis, indicating that the water in the fish was about 95 per cent saturated with salt. The samples were cut from the

thick part of the fish and the edges coated with paraffin wax, in the same manner as with the kench cured samples. Duplicates of the skin surface, razor cut surface, and the natural split surface of the fish were dried. A razor cut sample was placed on the recording balance. Pieces were dried in both the large and the small sections of the tunnel thus providing for simultaneous experiments at two velocities.

The results have been plotted on semi-logarithmic scale. Table I shows the fractional free water content after 10 hours and 48 hours drying, for the various air conditions. As in the case of the kench cured cod samples, the fractional free water content after 550 minutes of drying, plotted against time, gives a straight line relationship.

TABLE I. The effect of temperature at a relative humidity of 50 per cent and an air velocity of 100 cm. per second.

Type of surface	Air temperature		Fractional free water after drying time of:	
	(°C.)	(°F.)	10 hr.	48 hr.
Original, split	32.2	90	0.815	0.735
	29.4	85	0.820	0.717
	15.6	60	0.880	0.850
	10.0	50	0.895	0.870
Skin	32.2	90	0.821	0.680
	29.4	85	0.850	0.735
	26.7	80	0.868	0.765
	15.6	60	0.925	0.845
	10.0	50	0.920	0.850
Razor cut	32.2	90	0.820	0.800
	29.4	85	0.855	0.800
	26.7	80	0.815	0.600
	15.6	60	0.860	0.660
	10.0	50	0.900	0.800

TEMPERATURE

From the table it is evident that the higher the dry-bulb temperature of the air the faster the fish dries, although between 15 and 26°C. (60 and 80°F.) the difference in drying rate is small. The maximum dry-bulb temperature which may be used in salt fish dryers, without cooking the fish, has been established by this and other work, at 26.7 to 27.8°C. (80 to 82°F.). The fish may be exposed to a slightly higher dry-bulb temperature for a few hours, but temperatures as high as 32°C. (90°F.) partially cook the fish in a very short time. If the period of drying is prolonged without press piling, that is piling the fish until the salt solution from the interior has wet the surface, the dry-bulb temperature should not exceed 24 to 25.5°C. (75 to 78°F.). The rate of drying slows appreciably when the temperature falls below 15°C. (59°F.).

Relative Humidity

An examination of table II shows that the optimum relative humidity depends upon the extent to which the fish are dried. If the fish are dried for a few hours only, for example 10 hours, a relative humidity below 45 per cent is desirable. On the other hand, if the fish are to be dried continuously for two days or more, the relative humidity should be 50 to 60 per cent. It will be noted that the optimum relative humidity for drying depends upon the type of surface of the fish exposed to the air stream. There are different optima for skin, razor cut, and split surfaces. The effect of dry-bulb temperature on the optimum humidity was not completely investigated, but the indications are that the optimum is about the same for high and low temperatures. For the skin surface of the fish, the optimum humidity at the end of 10 hours of drying is 47 per cent, and at the end of 48 hours, it is 55 per cent. For the razor cut surface, the optimum humidity at the end of 10 hours is 50 per cent, and has the same value at the end of 48 hours. The split surface has an optimum humidity of approximately 50 per cent.

Table II. The effect of relative humidity at an air velocity of 100 cm. per second.

Type of surface	Air temperature dry-bulb		Relative humidity	Fractional free water after drying time of:	
	(°C.)	(°F.)	(%)	10 hr.	48 hr.
Original, split	26	80	45	0.850	0.790
			55	0.875	0.660
	15.6	60	35	0.910	0.850
			50	0.880	0.815
Skin	26.7	80	35	0.930	0.820
			45	0.850	0.750
			50	0.865	0.762
			55	0.880	0.715
			60	0.880	0.735

It is evident, then, that relative humidities from 45 to 55 per cent should be used in salt fish dryers. In the final analysis the appearance of the fish is a most important factor in determining the conditions which should be used in dryers. The relative humidity has an important bearing on the surface appearance. If humidities higher than 55 per cent are used, a rough, undesirable surface is formed. Therefore, it is recommended that at the start of the drying period, the relative humidity should be maintained at 50 per cent or lower. This value may be increased after the initial dried surface on the fish is formed.

When salt fish dryers were first installed, it was generally believed that the lower the relative humidity, the faster the fish would dry. In winter time, low relative humidities are readily procured, and humidities as low as 25 per cent were used in some commercial dryers. It soon became evident that the rate of drying at these low humidities was slower than at relative humidities of 40 to 50 per cent, which were then recommended. The results of experiments with the cut samples support this fact. Relative humidities of 35 per cent form a salt

crust on the surface very quickly and inhibit drying. Thus, the rate of drying at 35 per cent is less than the rate at 60 per cent. The type of surface produced, however, at the low humidity is not so rough as it is at 60 per cent relative humidity.

Surface Exposed to Air Stream

As has been noted in the case of samples from kench cured cod, the type of surface exposed, skin, razor cut, split surface, or nape, has a distinct effect on the rate of drying. The data for pickle cured cod are shown in table III. When the work on salt fish was started, it was presumed that the fish does not lose moisture to any appreciable extent through the skin surface. It is evident from table III that, on the contrary, the skin surface dries approximately one-half as fast as a split surface of the fish, or a surface cut with a razor. The ratio between the drying rates of the two surfaces depends to some extent on the dry-bulb temperature and the relative humidity used. If the relative humidity is increased, the rate of drying of the skin surface is practically the same as that of the split surface. At lower relative humidities, the rate of drying at the skin surface falls to about one-half the value of that at the split surface. No extensive experiments were carried out with the nape or peritoneal lining of the fish belly cavity but it appears that the rate of drying through this membrane is slower than through any other surface investigated.

Table III. The effect of different surfaces at an air velocity of 100 cm. per second.

Type of surface	Air-temperature dry-bulb		Relative humidity	Fractional free water after drying time of:	
	(°C.)	(°F.)	(%)	10 hr.	48 hr.
Split	15.6	60	35	0.912	0.850
Skin				0.918	0.860
Razor cut				0.890	0.780
Nape				0.930	0.890
Skin	26.7	80	35	0.925	0.825
Razor cut				0.890	0.702
Split	32.2	90	50	0.818	0.735
Skin				0.818	0.695
Razor cut				0.845	0.805
Split	15.6	60	50	0.887	0.815
Skin				0.930	0.858
Razor cut				0.858	0.660
Skin	26.7	80	50	0.865	0.765
Razor cut				0.818	0.615
Split	26.7	80	55	0.875	0.660
Skin				0.875	0.712
Razor cut				0.842	0.660

Air Velocity

The minimum air velocity has been fairly well established by Notevarp and Pillgram-Larsen (1935) at 100 cm. per second (200 feet per minute). Experiments with cut samples support this conclusion, no appreciable difference being found in the drying rates of samples at air velocities of 125 cm. per second (250 feet per minute) and 225 cm. per second (450 feet per minute). During the first 10 hours of drying green fish, it is recommended that the air velocity be maintained at 100 to 125 cm. per second (200 to 250 feet per minute). This value may be decreased toward the end of the drying period. Cooper and Wood (unpublished data) showed that at air velocities of the order of 50 cm. per second (100 feet per minute) or less, the drying rate is so slow that salt crystals build upon the surface and form an undesirable salty crust (salt face).

WHOLE SALT FISH

The previous sections of this paper give the optimum air velocity, relative humidity and dry-bulb temperatures for cut samples. The only satisfactory method for finding if the same conditions apply to whole fish is by trial on large numbers of fish. Because of the experimental difficulties in working with large lots of whole fish at many air velocities, temperatures and relative humidities, experiments were made only at selected air conditions, to check the previous findings. Both the turbo type dryer (Weisselberg and Weisselberg 1944) and the experimental tunnel were used. The humidity was controlled at any desired value by means of aluminium oxide (Lectrodryer, manufactured by the Pittsburg Lectrodryer Corp., U.S.A.) or by steam as required. Air velocities in the experimental tunnel were closely controlled. Much swirl in air flow was evident in the turbo dryer, and the air velocity measurements in this equipment were only an average value. The temperature of the turbo dryer was controlled by varying the supply of steam to the heater installed on the inlet duct. Most of the experimental work was done on a 3,000 pound (1,360 kg.) lot of fish obtained from a local fish company. The fish had been split, brined and washed in the usual manner and press piled before drying. Some of the experiments were carried out with heavily salted fish which had been pickle cured at the Atlantic Fisheries Experimental Station. The samples of kench cured cod were bank fish obtained from a Lunenburg fish company. The results include some 84 separate experiments and, because of the voluminous data, are difficult to present concisely. These data will be supplied upon request to anyone interested.

Air Temperature

A comparison of the results of drying pickle cured cod under identical conditions with air temperature as the only variable, shows no appreciable difference in the drying rates for temperatures of 22.6°C. (73°F.) and 26.5°C. (80°F.). Those fish which were dried at 26.5°C. (80°F.) for more than 30 hours continuously were slightly cooked near the tails.

Relative Humidity

Experiments were carried out at relative humidities of 30, 50 and 55 per cent. The drying rates at relative humidities of 30 and 50 per cent are almost the same,

and the variation is within the limit of experimental error. Other conditions being equal, when the relative humidity rises above 50 per cent, the drying rate falls off. Lower relative humidities tend to dry fish faster at the start. The higher humidities increase the drying rate near the end of the drying period (40 hours). If the drying is to be carried out in two stages, with intermediate press piling, it will be advantageous to keep the relative humidity at about 40 per cent. If the drying is to be continuous, a relative humidity of 45 to 50 per cent should be maintained.

Air Velocity

When the air velocity falls below the recommended value of 100 to 125 cm. per second (200 to 250 feet per minute) the drying rate is noticeably decreased. This confirms previous work which set the minimum air velocity for salt fish dryers at 100 cm. per second (200 feet per minute). Air velocities higher than 100 cm. per second (200 feet per minute) have little influence on the drying rate except when the relative humidity exceeds 50 per cent. Then the velocity becomes an important factor. This relationship may be expected. As the drying potential is decreased by raising the relative humidity, the effect of air velocity becomes more pronounced.

Press Piling

Two similar lots of fish were dried under identical drying conditions, one lot being dried continuously while the other was press piled between drying periods. With one press piling, fish were dried to a moisture content of 75 per cent in 50 hours, while it required 70 hours for continuously dried fish to reach this moisture content. Press piling is especially effective with large fish and more than one press piling may be necessary to dry them within a reasonable time. In practice, the decision whether to press pile or not will depend upon the local operating costs of handling the fish and the capacity of the dryer.

Cure

The results of the experiments in drying whole fish show little difference in the drying rates of kench or pickle cured cod. Kench cured fish, in general, have a lower initial moisture content, but the rate of moisture loss from the two types of cure is approximately the same.

Washing

Heavily salted pickle cured fish were soaked in fresh water for two hours before drying, and compared with unwashed samples. The washed fish dried to 73 per cent moisture in about 12 hours, while the unwashed sample required 75 hours. With large fish, the effect of washing was not so pronounced, although the drying time for the washed large fish was about one-half that of the unwashed sample. It would appear that it is desirable to dry heavily salted fish as soon after washing as possible, before the salt can diffuse to the surface of the fish, and lower the vapour pressure of the water on the surface. In practice, it is customary to press pile for several days after washing to smooth the rough surfaces. If an increased drying capacity is necessary, it may be desirable to dry directly

after washing and depend upon subsequent press piling to correct surface irregularities.

The effect of wetting agents was studied. Fish were washed in 0.1 per cent solution of aerosol, diamylester of sodium sulfosuccinic acid and in 0.1 per cent solution of igepon T., $C_{17}H_{33}CONHC_2H_4SO_3Na$. Drying results were the same as those for fish washed in water only.

Time of Drying

Medium size codfish, kench cured, having an initial moisture content of 130 per cent, dry basis, (56.5 per cent wet basis) may be dried under the recommended air conditions to 75 per cent moisture, dry basis, (43 per cent, wet basis) in about 40 hours, with one or two press pilings. Small fish may be dried continuously in 25 to 30 hours. Extra large cod with two press pilings, will require about 60 hours. This results in a moisture loss of 23 per cent of the total weight of the fish.

The most important factors influencing the drying time of heavily salted cod are the size of the fish and the initial moisture content. If the moisture content is as high as 150 per cent, the drying times given above will reduce the water content only to 92 per cent, dry basis, (48 per cent, wet basis). Under this condition, the drying time for medium size fish must be extended to 50 or 60 hours to reduce the moisture content to 75 per cent, dry basis (43 per cent, wet basis).

THEORY

A satisfactory theory of salt fish drying should explain the following facts:

1. Under optimum conditions of air temperature and relative humidity, the drying rate of salt fish is constant for air velocities in excess of 100 cm. per second (200 feet per minute). At velocities less than 100 cm. per second, the rate of drying decreases perceptibly.

2. A dry-bulb temperature variation between 15.6 and 32.2°C. (60° and 90°F.) has very little effect on the rate of drying.

3. The optimum relative humidity for drying salt fish is from 45 to 50 per cent. Fish will dry slower as the relative humidity departs from this region.

4. There is a definite break in the drying curve at six to ten hours of drying. The initial rate of drying constantly decreases until it reaches this break. Subsequent drying follows an almost constant rate.

5. At low drying potentials, salt crystals gather to form a rough, pebbly surface on the fish. During extremely slow drying at very low air velocities white needle-shaped structures may cover the surface.

6. Press piling of the fish, between drying periods, accelerates the rate of drying.

The drying curves on cut samples indicate quite clearly that after 10 hours, when the typical salt crust is formed on the surface, the type of drying of the fish is one in which air conditions are the controlling factor. They show that the diffusion of the water from the thick parts of the fish is not the limiting factor in controlling the rate of salt fish drying. As has been apparent in all this work, both on cut samples and on whole fish drying, the extent of the surface of the fish exposed to the air stream is the most important factor. The above discussion

applies when the fish are dried under optimum conditions. If the fish are dried at low air velocities or high humidities, the migration of the water to the surface of the fish may be the controlling factor, and hence the thickness of the sample will have an influence on the drying rate.

A physical examination of the salt crust formed on fish shows that it consists largely of salt crystals intermixed with a small amount of protein or salt free fish solids. As the fish dries this salt crust thickens slightly, but the centre of the fish always appears to have a translucent quality which gradually decreases in extent as the fish are dried. The results of the determination of distribution of water in the fish (unpublished data) confirm the fact that a salt crust is formed at the surface. A typical analysis of this crust gives 80 per cent salt, 10 per cent protein, and 10 per cent water. Layers underneath the crust analysed for salt and water show a steadily increasing water content until at depths below 2 mm. the ratios of salt to water reach a constant value, the water in this whole portion being just saturated with salt. The salt and water distribution experiments also demonstrate clearly that the salt solution in the fish moves to the surface along the capillaries in the muscle under the influence of surface tension. The movement of the salt and water in fish or other biological material follows the laws of capillary flow and not those of molecular diffusion.

These considerations explain why the rough surface is formed on salt fish at low drying potentials. After water is removed slowly by evaporation from the surface of the fish the salt solution migrates along the capillaries, through the salt crust at the surface. There, an ever-increasing thickness of salt crust is built up similar to the manner in which salt solutions "creep" when evaporation occurs from a solution in an open vessel. On the other hand, when the drying potential is high evaporation occurs in or below the salt crust formed on the fish. This subsurface evaporation occurs at a zone which gradually retreats as the fish is dried. Excessively low humidities, then, dry the salt protein crust to such an extent that the water vapour is not able to diffuse through the layer. These facts account for the slow drying rate at low humidities.

The results of the water-salt distribution experiments with the cut samples of kench cured cod, indicate that during the first 6 to 10 hours of drying, a parabolic distribution of moisture is set up in the sample of material.

Air velocities above 100 cm. per second (200 feet per minute) or a change in dry-bulb temperature have no influence on the drying rate. This fact supports the theory that salt crust is formed on the sample, and that drying must consist in evaporation of the liquid water into vapour below this crust. Apparently variations in relative humidity affect the equilibrium moisture content in the salt crust surface, and hence change the permeability of this crust to the diffusion of water vapour through it. Changes in relative humidity influence the drying rate for this reason.

Press piling of salt fish permits the moisture and salt solution at the centre of the sample to migrate to the surface along the capillaries during the period of storage. The surface is rewetted with the salt solution, and when the sample is placed in the air stream drying proceeds again at a relatively fast rate, similar to the rate of drying of salt fish out of pickle.

SUMMARY

The optimum conditions for the drying of heavily salted cod fish, cured either under saturated brine or with dry salt in piles, are as follows: air velocity, 125 cm. per second (250 feet per minute), air temperature between 15.6 and 26.7°C. (60 to 80°F.), and relative humidity of 45 to 55 per cent. Under the above conditions initial drying is a surface phenomenon, the brine saturated or nearly so, moving to the surface along the capillaries in the muscle under the influence of surface tension. When drying has proceeded for six hours evaporation occurs in or below the salt layer built up on the surface, resulting in a slower rate of drying.

Press piling effects a thorough wetting of this surface salt crust, permitting an increased drying rate until the layer is re-formed.

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Counts of Gill Rakers and Pyloric Caeca in Pink Salmon

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ABSTRACT

Counts of gill rakers and pyloric caeca on pink salmon (*Oncorhynchus gorbuscha*) from different rivers in British Columbia overlap in range to such an extent that it would be impossible with any degree of accuracy to assign isolated individuals from an ocean population to a distinct "breeding group" on the basis of these characters. While the comparison of the averages of each character demonstrates significant differences between separate rivers in the same year in some cases, in others no such difference is noticeable. The averages for the characters do not consistently show differences between populations of two succeeding years which are known to be distinct, e.g. those of 1940 and 1941.

The counts of gill rakers and pyloric caeca submitted herein for the pink salmon (*Oncorhynchus gorbuscha*) were made for the definite purpose of determining whether there are any significant differences in these characters between various "breeding stocks" or populations, and whether such differences were sufficiently large to enable recognition of specific groups of salmon in a mixed ocean population.

McGregor (1923) concluded after examining numerous king salmon (*O. tschawytscha*) that: "the results of our studies of the ova, pyloric caeca, gill rakers, and vertebrae would appear to support the belief that the Klamath and Sacramento races of king salmon possess anatomical differences of sufficient magnitude to enable careful workers to recognize individuals of the two races." More recently Parker (1943) and Townsend (1944) have discussed the efficacy of employing counts of pyloric caeca, gill rakers and vertebrae in separating populations of king or chinook salmon in California and in the Columbia river drainage. Both authors have shown that the method would be of little use to the conservationist because of the great degree of overlapping in counts between samples. Furthermore, while certain significant variations have been shown to exist between the means for a given character in groups from different rivers, the final decision as to their complete reliability in indicating separate and distinct stocks requires much more carefully planned and continuous sampling over a succession of years.

The pink salmon (*O. gorbuscha*) appears to offer a special opportunity, as compared with the other species of the genus, for the investigation of this problem in perhaps its simplest form. Since all individuals in the commercial catches and on the spawning grounds are in their second year, it follows that fish taken

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in one season must be genetically distinct from those captured in a succeeding one, e.g. 1940 and 1941, since they could not have had common ancestors. There can be no heterogeneity in respect to age such as might be encountered in the king or chinook (*O. tschawytscha*), the sockeye (*O. nerka*) and the chum (*O. keta*). In addition adult pink salmon are all approximately the same size.

During the autumn of 1940 samples of gill rakers and pyloric caeca were gathered from spawned-out fish in seven rivers on the Queen Charlotte islands, viz.—McClinton creek, Deenan river, Yakoun river, Mammon river and Detlamen river, all tributaries to Masset inlet on Graham island, and Haan creek and Palent creek on Moresby island. During the autumn of 1941, additional collections were secured from Morrison creek, tributary to the Courtenay river on the east coast of Vancouver island, and from the Harrison river, Chehalis river, Lorenzetti creek and Sucker creek draining into the lower Fraser.

The material was treated and handled in almost the same way as that gathered by Townsend (1944). Samples were preserved in strong formalin immediately upon collection. All observable gill rakers on the first gill arch on the left side of the fish were enumerated. After sufficient hardening, the caeca were removed one by one with forceps from the pyloric area of the stomach and counted. Branched caeca, though rarely encountered, were considered as one.

The range in gill raker counts on 1,045 individuals is from 24 to 35 with the mode at 30. This is slightly larger than the 27 to 33 given by Clemens (1935) and the 27 to 35 submitted by Schultz (1936). In all, however, only eight individuals are included in the 24, 25, 26, 34 and 35 groups.

All possible comparisons have been made between the means of the counts of gill rakers for the different streams. A difference is assumed to be significant when it is greater than 3.9 times its probable error ($P < 0.009$). On this basis the Haan creek average differs only from those for Morrison creek and Harrison river at the bridge. The Morrison creek mean is, in addition, significantly different from those for Lorenzetti and Sucker. The Harrison river pinks taken at the bridge differ from those gathered in Lorenzetti creek, Sucker creek, Haan creek and Palent creek. The average for pinks captured in the Harrison at the rapids differs only from that of Palent creek. The Lorenzetti figures differ from those for the Harrison river at the bridge and Morrison creek. The average for Deenan river differs from that for Palent creek. That for the composite sample from Masset inlet (McClinton, Deenan, Yakoun, Mammon and Detlamen) differs greatly from that for Moresby island (Haan and Palent creeks).

Comparing the samples taken in the same year, it may be concluded that differences do exist amongst collections from various tributaries of the lower Fraser river. There appears to be no significant variation in respect to gill rakers between the groups from Masset inlet. Haan and Palent creeks are similar but if combined differ radically from the composite collection in Masset.

Since it was felt that the application of analysis of variance to the figures might give a clearer approach to the problem, this was applied. The justifiable deductions were similar to those already drawn, viz.—(1) all samples could not have been taken from the same population, (2) the Fraser river collections could not all have been drawn from the same population and some of these differ from that taken at Morrison creek, and (3) while the runs to Masset inlet are homo-

geneous, they differ from those to Moresby island (Haan and Palent creeks) which also show homogeneity *inter se*.

The ranges in the counts for the gill rakers in the samples from the various streams overlap to such an extent that it would be impossible with any degree of accuracy to segregate individuals from various "breeding groups" on the basis of the character.

The range in the number of pyloric caeca for the 981 specimens extended from 99 to 195 with the mode occurring between 131 and 135. These data therefore change greatly the lower limit (165) given by Clemens (1935) and indicate that the mode and average are much smaller than previously indicated.

TABLE I. The numbers of gill rakers in pink

	Vancouver is. (1941)	Fraser r. tributaries (1941)				
Number of gill rakers	Morrison cr.	Harrison r. at bridge	Harrison r. at rapids	Chehalis r.	Lorenzetti cr.	Sucker cr.
24				1		
25						
26					1	
27			1	2	3	
28	1		4	5	4	5
29	13	4	13	17	16	15
30	23	4	29	26	36	23
31	37	12	24	33	27	14
32	21	5	14	18	14	4
33	3	1	2	4	2	1
34	1		1			
35			1			
Total.....	99	26	89	106	103	62
Mean.....	30.78	30.81	30.48	30.40	30.23	30.00
P.E. of mean.	± .075	± .14	± .10	± .09	± .09	± .09

Comparison of the averages of various streams, using the same test of significance as in the case of gill rakers, reveals quite different conclusions. Haan creek is distinct from all others. The Morrison creek average differs from that for Sucker creek. The mean for Harrison river pinks taken below the bridge differs from those for the Chehalis river, Sucker creek, McClinton creek, Deenan river and the Yakoun river. The average for pinks from the Harrison at the rapids is different from that for the Chehalis. The Lorenzetti creek figure differs from those for the Chehalis, Sucker, McClinton, Deenan and Yakoun.

The pyloric caeca, therefore, in addition to indicating heterogeneity in the populations in the lower Fraser river and homogeneity in Masset inlet show that

Haan creek pinks are different from those of Palent creek, i.e. possible heterogeneity in the Moresby island populations.

As in the case of the gill rakers, the ranges for the counts of pyloric caeca from the various streams overlap to such an extent that isolated individuals could not be segregated accurately.

DISCUSSION

Comparison of the average counts of gill rakers and pyloric caeca from samples of pink salmon taken from different rivers in British Columbia in the same year, viz.,—Queen Charlotte island streams in 1940, and the Fraser river tribu-

salmon from various rivers in British Columbia.

Queen Charlotte is. streams (1940)							
McClinton cr.	Deenan r.	Yakoun r.	Mammon r.	Detlamen r.	Haan cr.	Palent cr.	Total
							1
							1
1		1			2		10
3	2	5	7	1	1	9	47
29	13	20	16	13	5	17	191
42	13	35	30	26	8	30	325
43	13	27	26	16	7	16	295
16	15	9	15	7	1	7	146
2	2	2	3	1			23
		1	1	1			5
							1
136	58	100	98	65	24	79	1045
30.32	30.55	30.22	30.40	30.34	29.83	29.94	
± .09	± .11	± .08	± .087	± .091	± .17	± .083	

taries and Morrison creek in 1941, have revealed that in some cases significant differences are demonstrable while in others no variation appears to exist. Two deductions are therefore possible:—(1) certain rivers have distinct and separate populations while many others are frequented by the same race, or (2) all rivers may have separate populations but in some cases the characters used have failed to reveal the distinction.

Owing to the fact that all fish in the samples were in their second year, there is no doubt that the fish collected in 1940 are genetically distinct from those of 1941. Comparison between these years shows significant variations in certain instances but not in others. Since one cannot overlook the possibility that two

separate and distinct populations might be morphologically similar, the only inference which one can draw from these findings is that counts of pyloric caeca and gill rakers do not consistently reveal differences between distinct populations.

Because a moderately high degree of correlation exists between the average numbers of gill rakers and the average numbers of pyloric caeca in the samples (correlation coefficient -0.60 ± 0.12), it must be accepted that the characters

TABLE II. The numbers of pyloric caeca in pink

	Vancouver is. (1941)	Fraser r. tributaries (1941)				
Number of caeca	Morrison cr.	Harrison r. at bridge	Harrison r. at rapids	Chehalis r.	Lorenzetti cr.	Sucker cr.
96-100						1
101-105			1	1		2
106-110	1		3	1	1	2
111-115	2	1	3	2	3	
116-120	6	2	6	9	9	5
121-125	17		9	9	9	7
126-130	11	5	6	17	11	8
131-135	11	3	16	13	9	7
136-140	9	3	7	6	17	4
141-145	12	2	9	8	9	11
146-150	11	2	8	5	10	9
151-155	5	3	5	3	8	1
156-160	5		4	5	7	1
161-165	3	1	4		3	1
166-170	2	1			1	
171-175		2			2	
176-180					1	
181-185		1			1	
186-190						
191-195			1			
Total.....	95	26	82	79	101	59
Means.....	137	143	136	133	139	131
P.E. of means	± 0.91	± 2.4	± 0.7	± 0.92	± 1.02	± 1.14

vary coincidentally. Since, however, such correlation is not perfect, it is not surprising that in some cases gill rakers indicate significant differences between two selected groups while pyloric caeca fail to reveal any variation.

Ginsberg (1938) has proposed a simple method for the comparison of related populations. It is perhaps interesting to apply this to the case where the most extreme difference between the sample averages is indicated, viz., Morrison creek vs. Sucker creek. For the gill rakers a measure of divergence of 66 per cent is

shown which according to Ginsberg's classification indicates racial status. For pyloric caeca the measure of divergence is 59 per cent or just under the racial status set at 60 per cent. On the basis of this mathematical conception, therefore, counts of gill rakers and pyloric caeca in pink salmon of distinct groups will indicate a complete range from no difference where little or no divergence exists, to racial status shown in the example above.

salmon from various rivers in British Columbia.

Queen Charlotte is. streams (1940)							
McClinton cr.	Deenan r.	Yakoun r.	Mammon r.	Detlamen r.	Haan cr.	Palent cr.	Total
							1
							4
3	3		2	2	5	3	26
4	5	6	4	3	2	7	42
10	7	5	6	6	1	6	78
24	9	18	10	7		7	126
16	6	18	11	8	2	8	127
24	6	13	13	15		8	138
15	4	14	8	10		6	103
11	6	14	14	4		13	113
11	2	10	14	5		10	97
4	2	2	7	5		5	50
4	2	2	6	1		1	38
2						3	17
	3	2		1		1	11
							4
							1
							2
				1			1
						1	2
128	55	104	95	68	10	79	981
133	131	133	136	134	114	136	
± 0.71	± 1.4	± 0.76	± 0.89	± 1.2	± 1.2	± 1.1	

The great degree of overlapping in the ranges between all samples demonstrate conclusively that any attempt to assign an isolated individual on the basis of such enumerations to a definite "breeding group" or distinct population would be very inaccurate if not impossible.

If a mixed ocean population is interpreted as a random mixing in the sea of individuals from many streams, it is very doubtful whether pink salmon from specific rivers could be segregated therefrom on the basis of such characters even

if much more extreme variations existed than at present indicated. Such an accomplishment would be possible only in special cases where the counts for one river were all very high and for the other very low.

If a mixed ocean population is accepted as meaning schools of salmon in close proximity each preserving to some extent their local identity, i.e. each school in the main bound for a separate stream, there is a possibility of rough separation at least to the extent of indicating in some cases the general area to which the run is headed. Such a procedure would, however, be dangerous unless much more data were obtained to establish the extent of variation from cycle to cycle, etc.

SUMMARY

The counts of gill rakers and pyloric caeca recorded herein for the pink salmon (*O. gorbuscha*) in British Columbia, have extended the ranges previously recorded for the characters.

Comparison between the averages for counts on samples from various rivers in the same year shows that significant differences may exist in some cases and not in others. Comparison of averages for samples taken in successive years, e.g. 1940 and 1941, where the fish are known to be genetically distinct demonstrates variation in only some instances. These characters, therefore, will not suffice in every case to separate populations of pink salmon.

In certain instances comparison of gill raker averages will reveal a significant variation between rivers while means for pyloric caeca are not statistically different.

The great degree of overlapping in the ranges of the counts makes it impossible with any accuracy to assign an isolated individual to a definite "breeding group" or distinct population.

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Migration of Salmon Parr

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ABSTRACT

Some salmon (*Salmo salar*) parr in a stream descend from the spawning areas, particularly during freshets, to populate lower waters, such as lakes and fresh or brackish parts of estuaries, although killed by full sea water. Thence they ascend and populate available streams variable distances, depending upon conditions such as falls.

Since "parr" is the name given to salmon during the time (1 to 8 years) that they remain in their native streams before transforming into smolts and migrating seaward, it would at first seem wrong to apply to any of their movements the term "migration." This term seems, however, to be applicable to changes in residence or habitat at any stage in the life history of the fish.

DOWNSTREAM MOVEMENT

That some of the parr in a river may move downstream is shown by finding them dead in the sea in relation to the river outflow, as for the Matamek river, Quebec, in late July, 1931, and the St. Croix river, N.B., in September, 1933 (Huntsman and Hoar 1939). The former was not in relation to a freshet, but the river water flowed over the beach into the sea on a straight coast, that is, without an estuary. The latter was after an extremely heavy freshet. At the Garden pool, about 6 miles (1 mi. = 1.6 km.) above the head of tide in the Northeast Margaree river, N.S., planted underyearling salmon that had occupied a shallow, stony rapid for some weeks, disappeared with a heavy freshet [100 cu. ft. rising to 2,000 cu. ft. (1 cu. ft. = 28.3 l.) per sec.] in late August, 1935, and failed to be found there again after the freshet subsided. It may be noted that during the flood planted fry were found to be holding their position in Salt brook, a tributary in which the water was far from becoming so deep or the current so strong as in the river. The numbers of three different sizes of parr, as observed at night along a certain part of the shore of the pool, decreased to nothing with full development of the freshet, and as it passed away the numbers gradually came back to the original amounts, except for the planted size, which remained few. If, as is presumed, the freshet, which locally was a torrent, shifted all the parr downstream, the native fish at least either returned or were replaced by others, conceivably from farther up the river.

In trapping two brooks tributary to Moser river, N.S., in 1942, P. F. Elson

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(unpub.) found that both underyearlings and yearlings descended without relation to freshets (rise and fall in water) as well as in relation to them, but the proportion with freshets was from 75 to 77% of the total in each of three cases (underyearlings for one brook and larger parr for both brooks). So far as could be seen, most of these failed to return upstream, and the occurrence of parr in Mill lake, into which these brooks discharge, is doubtless to be explained by such descent.

Salmon parr were found on one occasion to be numerous in dead water at the mouth of Glenmore brook on the east side of lake Ainslie, Margaree river, N.S. The brook is well populated with young salmon, and it or other tributaries of the lake must have furnished these parr to the lake.

The inner part of the Margaree estuary was found on August 15, 1935, to be well populated with both underyearling and yearling parr. That some occur at times even in the outer part of the estuary, which is ordinarily altogether brackish or salt, that is without fresh water (H. M. Rogers unpub.), is indicated by the presence of bones of yearling parr in the pellets of kingfishers feeding along this part of the estuary (White 1937). The origin of the estuarial parr cannot be stated, although it may be presumed that they were from spawning grounds above the head of tide.

It may be concluded that lakes and inner fresh or brackish parts of estuaries may become populated with parr, through descent of a portion of the stock in streams.

ASCENT OF TRIBUTARIES

The small tributaries of the main part of the Margaree river (below the Forks), $2\frac{1}{2}$ miles in length, and of its estuary, $5\frac{3}{4}$ miles in length, do not seem to have any native salmon. Streams quite as small as these have been found elsewhere to contain native salmon, but only in association with beds of clean gravel, which these lack. They resemble in this respect the streams flowing into lake Ainslie from the west, which contain young trout but no young salmon. Residents have no knowledge of large salmon entering them, and we have found no evidence of salmon redds in them. The numbers of square miles in their watersheds are given as calculated roughly from the map by Hugh Fletcher for his report on the geology of the region, published in 1884 by the Geological and Natural History Survey of Canada.

On August 23, 1935, young salmon were found in Bochan (Gaelic for "ghost") brook [$\frac{3}{4}$ sq. mi. (1 sq. mi. = 2.6 sq. km.) watershed], which enters the estuary from the west a mile and a half below the head of tide. Underyearlings were quite numerous in the two larger of the branches by which the brook discharges into the estuary in relation to a delta, but they decreased in number going up and there was only an occasional and large one above the delta and none found beyond 120 feet (1 ft. = 3 dm.). Yearlings occurred in suitable places all the way along to, but not beyond, a fall $17\frac{1}{2}$ in. (1 in. = 2.5 cm.) high at the road culvert, 330 feet from the mouth, there being a second fall of slightly greater height (19 in.) at the upper end of the culvert. Trout underyearlings and older fish were generally distributed above as well as below the culvert. It was considered probable that this brook was being populated by young salmon ascending from the estuary.

This result for Bochan brook was confirmed on October 9. On October 10 the Gillis brook ($1\frac{1}{2}$ sq. mi. watershed), entering the river from the west about a mile above the head of tide, was examined. It had rather continuous rapids, but no falls, from the short delta up as far at least as one-quarter of a mile, and both underyearlings and larger parr were found all the way along it. On the same date the Widow Lord's brook (5 sq. mi. watershed) which enters the estuary from the west about $2\frac{3}{4}$ miles below the head of tide, was found to have underyearling and larger parr from the mouth up a distance of 1,400 feet, but not farther. No salmon were found in the small Duck Cove brook (3/8 sq. mi. watershed) about two miles farther out and discharging into the outer brackish or salt part of the estuary. However, two underyearlings were found in the lower sluggish part of a brook that enters the estuary on the east side at the salmon-retaining pond, half-a-mile above, where also the water is ordinarily brackish. Rapid brooks (Blanchard's and Gilbert's) on the east side discharging into the brackish portion of the estuary between the last and the Widow Lord's brook showed good populations of both underyearlings and larger parr as far up as examinations were made, some distance above the road bridge in each case. These findings indicated that the streams tributary to the lower part of the river and estuary were being populated by migration upward from their mouths, but not very effectively, if at all, where the water became decidedly salt.

Attempts were then made to trace the presumed ascent by underyearlings of Bochan and Widow Lord's brooks. It had been found that in the Margaree river they emerge from the redds about the end of June. On July 7, 1936, yearlings were found in Bochan brook as far up as the road culvert, but no underyearlings, and in the river near the mouth of the brook only yearlings were found. On July 27, underyearlings were seen in numbers (over a dozen in one school) in the river near the mouth of the brook and heading into the brook outlets, chiefly the middle one. The latter was the largest and flowed out over a bank, thus keeping near the surface and making an evident turbulent current farther into the river than did the other two outlets. On making the outlets muddy, the middle one was seen to flow out farther and to become clear more quickly than the others. The brook water, being colder, finally sank to the bottom below the river water. No underyearlings were above the delta, but yearlings were there as well as in the river. In 1937, none were found in the brook by Mr. W. S. Hoar on June 27, but examination on July 7 showed underyearlings in the north outlet for a distance of 36 feet and in the middle outlet for 27 feet.

On August 16, 1936, underyearlings were found in the Widow Lord's brook as far up as an old broken dam (1350 ft.), just above the second bridge, that is, not as far as in October 1935; but yearlings occurred as far up as some distance above the third bridge (1900 ft.), that is, far beyond where the young were found in 1935. In 1937, examination of the brook on June 28 from the estuary up to the highway or first bridge (550 feet) revealed only yearlings. On July 7, underyearlings were found nearly up to that bridge. On July 20 and 21, they were found as far as about 75 feet above the bridge, but no farther. In 1938, on July 9 examination revealed many underyearlings from the estuary for some distance up the brook but only one could be found on the very suitable bottom just below the bridge, while yearlings were generally distributed.

On August 16, 1936, Blanchard's brook (1 sq. mi. watershed) on the east side of the estuary a short distance riverward from the salmon-retaining pond was examined. Underyearlings were found in it up as far as 480 feet, while yearlings were at least as far as 1,000 feet upstream. In Gilbert's brook (1 sq. mi. watershed) just above on the same side, there were many underyearlings up as far as the road bridge, which is beside the estuary, but not above. At the bridge there was a fall of one foot, which was evidently preventing farther ascent, though yearlings had been found farther up.

It seems justifiable to conclude that soon after the young salmon emerge from the redds in the Margaree river, some of them pass down to the estuary and for several miles seaward even into the brackish portion, and work up the tributary streams, which are rather rapid, a distance which increases with time. Falls as high as one foot seem to stop the underyearlings, and as high as one-and-a-half feet also the yearlings.

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